REMARKS/ARGUMENTS

Claims 1-17 and 19-38 are active in this application. Claims 1-17, 19-22, 26-29 are drawn to the elected subject matter. Support for the amendments to Claims 13-15 and 17 on page 5, first paragraph and page 9, first paragraph. Support for the amendment to Claim 16 is found on page 5, lines 11-15. Support for Claim 27 is found on page 5, first paragraph, lines 26-30, and page 6, last paragraph. No new matter is added by these amendments. Favorable reconsideration is requested.

An abstract is provided and the specification on page 12 has been amended in accordance with the Examiner's request.

The rejection of Claims 1-6, 10-22 and 26-28 under 35 U.S.C. § 102(a) over Nakagawa et al. is respectfully traversed.

Nakagawa was published on June 20, 2001. The present application claims priority to a provisional application which was filed on March 29, 2001 and two German applications, DE 142052.4 filed August 26, 2000 and DE 10110053.1 filed on March 2, 2001. To perfect priority to these German priority documents, Applicants submit herewith certified English translations of the same. Accordingly, reconsideration and withdrawal of this rejection is requested.

The rejection of Claim 16 under 35 U.S.C. § 102(b) over GenBank Accession No. AE001274 is respectfully traversed.

While this GenBank entry describe the DNA sequences with 23 consecutive nucleotides from nucleotides 926 to 948, this sequence does not describe the isolated polynucleotide now claimed in amended Claim 16. Accordingly, withdrawal of this ground of rejection is requested.

The rejection of Claim 17 under 35 U.S.C. § 102(b) over either GenBank Accession No. AF 186371 or U18263 is respectfully traversed.

Claim 17, as amended herein defines the hybridization conditions to SEQ ID NO.: 1, is at least 70% identical to SEQ ID NO.: 1 and which includes protein with oxyR transcriptional regulation activity. As the sequences in the two GenBank entries have a 54 and 55% similarity to SEQ ID NO.: 1, these Gen Bank sequences do not anticipate Claim 17. Accordingly, reconsideration and withdrawal of this ground of rejection is requested.

The rejection of Claims 2 and 18 under 35 U.S.C. § 112, second paragraph is respectfully traversed.

Applicants submit that the oxyR transcriptional regulator activity is a well known and defined term in the art. For reference, the Examiner's attention to the attached publications which describe oxyR transcriptional regulation activity from E. coli. Thus, the phrase "oxyR transcriptional regulator activity" is deemed to be clear and definite to one of ordinary skill in the art (see M.P.E.P. § 2173.02).

The rejection of Claims 13-17 under 35 U.S.C. § 112, first paragraph ("written description") is respectfully traversed.

Claims 13-17 have been amended to define that the polynucleotides which have identity and/or hybridize under stringent conditions to SEQ ID NO.: 1 also must encode a protein with oxyR transcriptional regulation activity. Pointing to the Examiner's attention to the discussion on page 9 it is clear that the application describes SEQ ID NO.: 1, which is a species of the claimed genus, the common characteristic of each of the claimed molecules, i.e., a structural similarity to SEQ ID NO.: 1, and the polynucleotides encode a protein with a particular activity.

As a result, Claims 13-17 are adequately described and as such withdrawal of this ground of rejection is requested.

The rejection of Claims 13-17 under 35 U.S.C. § 112, first paragraph is respectfully traversed.

This rejection is believed to be traversed as the polynucleotides included in Claims 13-17, which have been amended to couple the structural limitations relating to SEQ ID NO.: 1 along with its ability to encode a protein having a specified enzymatic activity. As already cited by the Examiner, attention is again drawn to In re Wands, 8 USPQ 2d 1400, 1404 (Fed. Cir. 1988), which states: "Time and difficulty of experiments are not terminative if they are merely routine." (see also M.P.E.P. § 2164.06). Again citing In re Wands, M.P.E.P. § 2164.06 states: "The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed."

Applicants point to pages 5-9 of the present specification which provides copious amounts of guidance and definition to make and identify polynucleotides that fall within the scope of Claims 13-17. Applicants also point to Examples 1-5 (pages 14-21) in which an actual isolation example is provided. Accordingly, reconsideration and withdrawal of this ground of rejection is requested.

The rejection of Claim 27 under 35 U.S.C. § 112, first paragraph is obviated by amendment. As amended herein, Claim 27 defines that the bacterium comprises an overexpressed polynucleotide. Accordingly, withdrawal of this ground of rejection is requested.

The rejection of Claims 27-28 under 35 U.S.C. § 112, first paragraph is addressed by amendment. As amended herein, Claim 27 is amended to define a coryneform bacterium which contains a polynucleotide selected from three options. Accordingly, withdrawal of this ground of rejection is requested.

The rejection of Claim 29 under 35 U.S.C. § 112, first paragraph is respectfully traversed.

Applicants point the Examiner's attention to the deposit receipt of DSM 13457 submitted with this application on August 27, 2001. In addition, Applicants state that all restrictions on the public availability of the deposit of microorganism will irrevocably and without condition be removed upon the granting of a patent from this application. Accordingly, withdrawal of this ground of rejection is requested.

The rejection of Claims 6 and 22 under 35 U.S.C. § 112, second paragraph is addressed by amendment.

The rejection of Claim 17 under 35 U.S.C. § 112, second paragraph is addressed by amendment.

The rejection of Claim 27 under 35 U.S.C. § 112, second paragraph is addressed by amendment.

The objection to Claims 2 and 18 is obviated by cancellation of the claims. The objections to Claims 5, 6, 21, 22 and 27-28 is obviated by correction of the bacterial name. The objection to Claim 12 is addressed by amendment, noting that the claim has been amended in accordance with the Examiner's suggestion, i.e., providing the full complement of the coding strand of SEQ ID NO.: 1. The objection to Claims 13-17 is obviated by amendment.

Finally, Applicants request that this application be passed to issuance.

Respectfully submitted,

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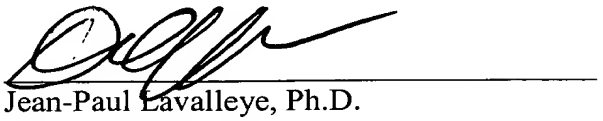

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- Storz, G., L.A. Tartaglia, B.N. Ames 1990. OxyR, a transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science 248:189-194
- Kullik, I., M.B. Toledano, L.A. Tartaglia, G. Storz 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. J.Bacteriol. 177:1275-1284
- Kullik, I., J. Stevens, M.B. Toledano, G. Storz 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. J.Bacteriol. 177:1285-1291
- Henderson, I.R., M. Meehan, P. Owen 1997. A novel regulatory mechanism for a novel phase-variable outer membrane protein of Escherichia coli. Adv. Exp.Med. Biol. 412:349-355
- Zheng, M., G. Storz 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation [see comments]. Science 279:1718-1721
- Gonzalez-Flecha, B., B. Demple 1997. Transcriptional regulation of the Escherichia coli oxyR gene as a function of cell growth. J.Bacteriol. 179:6181-6186
- Tao, K., K. Makino, S. Yonei, A. Nakata, H. Shinagawa 1989. Molecular cloning and nucleotide sequencing of oxyR, the positive regulatory gene of a regulon for an adaptive response to oxidative stress in Escherichia coli: homologies between OxyR protein an
- Tartaglia, L.A., G. Storz, B.N. Ames 1989. Identification and molecular analysis of oxyR-regulated promoters important for the bacterial adaptation to oxidative stress. J.Mol.Biol. 210:709-719
- Mukhopadhyay, S., H.E. Schellhorn 1997. Identification and characterization of hydrogen peroxide-sensitive mutants of Escherichia coli: genes that require OxyR for expression. J.Bacteriol. 179:330-338
- Zheng, M., B. Doan, T.D. Schneider, G. Storz 1999. OxyR and SoxRS regulation of fur. J.Bacteriol. 181:4639-4643

Transcriptional Regulator of Oxidative Stress-Inducible Genes: Direct Activation by Oxidation

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The *oxyR* gene positively regulates genes induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*. Purification of the OxyR protein showed that oxidized but not reduced OxyR activates transcription of oxidative stress-inducible genes in vitro. Conversion between the two forms of OxyR is rapid and reversible. Both the oxidized and the reduced forms of the OxyR protein are capable of binding to three diverse sequences upstream of OxyR-regulated promoters, but the interactions of the two forms of OxyR with the promoter regions are different. The results suggest that direct oxidation of the OxyR protein brings about a conformational change by which OxyR transduces an oxidative stress signal to RNA polymerase.

THE MECHANISMS BY WHICH CELLS SENSE ENVIRONMENTAL adversity and then transduce the stress signals into a change in gene expression are known for only a limited number of responses. MerR, a regulator of mercury resistance in *Escherichia coli*, is activated to induce mercuric reductase upon binding mercury (*1*). The *E. coli* Ada protein, which regulates the expression of genes in response to DNA methylation, is activated by the transfer of a methyl group from the DNA to the Ada protein (*2*). For other environmental stresses, the transcriptional regulator has been characterized, but little is known about how the environmental signal is transmitted to the transcriptional regulator. For example, the level of RpoH, a regulator of the heat shock response in *E. coli*, is increased after a shift to higher temperature (*3*), and the transcriptional activity of the heat shock factor in *Saccharomyces cerevisiae* is thought to be modulated by phosphorylation (*4*), but the actual sensors of heat shock are still unknown.

The cellular response to oxidative stress is of importance since reactive oxygen species including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxy radical ($HO^{\cdot}$), have been implicated as causative agents in several degenerative diseases (*5*). Reactive oxygen species can be produced by the incomplete reduction of oxygen during respiration, by exposure to radiation or to oxidation-reduction (redox) active drugs such as paraquat, or by release from macrophages in response to bacterial invasion (*5*). They can lead to damage of almost all cell components—DNA, lipid membranes, and proteins (*5*). Both prokaryotic and eukaryotic cells have inducible defenses to counter oxidative damage (*6–8*), but the mechanisms by which cells receive and respond to oxidative stress have not yet been elucidated.

The *oxyR*-controlled regulon of hydrogen peroxide–inducible genes in *Salmonella typhimurium* and *E. coli* has provided a model for studying the cellular response to oxidative stress. When bacterial cells are treated with low doses of hydrogen peroxide, the synthesis of at least 30 proteins is induced, and the cells become resistant to subsequent doses of hydrogen peroxide that would otherwise be lethal (*7–9*). The expression of nine of the proteins induced by hydrogen peroxide treatment is under the control of the *oxyR* gene (*8*). Strains carrying deletions of *oxyR* are unable to induce the nine proteins and are hypersensitive to hydrogen peroxide and other oxidants (*8*). Several of the proteins whose expression is regulated by *oxyR* have been identified and include catalase, encoded by *katG*, and an alkyl hydroperoxide reductase, encoded by *ahpC* and *ahpF* (*8, 9*).

Sequence analysis of the *oxyR* gene revealed that OxyR is a member of a large family of bacterial regulators that includes the *E. coli* regulatory protein LysR and the *Rhizobium* regulatory protein NodD (*10–12*). Like many other members of the LysR family, OxyR acts as both a positive and a negative regulator. OxyR is an activator of *katG* and *ahpCF* expression and negatively regulates its own expression (*10, 13*). The levels of the *katG* and *ahpCF* mRNA's are greatly increased in *oxyR* mutant strains that have constitutively high levels of the *oxyR*-regulated gene products suggesting that OxyR regulates at the level of transcription (*9, 13*). Extracts of strains that overproduce the OxyR protein protect regions upstream of the *oxyR*-regulated *oxyR*, *katG*, and *ahpC* promoters from deoxyribonuclease (DNase) I digestion although the protected sequences show very little sequence similarity (*13*). Bölker and Kahmann have also found that OxyR is a repressor of the *mom* gene (encoding a DNA modification function) of phage Mu (*11*). To elucidate the mechanisms by which bacterial cells sense oxidative stress and then induce a defense response, we purified the OxyR protein to homogeneity and studied its ability to bind *oxyR*-regulated promoters and to activate transcription in vitro.

No increase in amount or synthesis of OxyR after treatment with hydrogen peroxide. The expression of *oxyR*-regulated proteins as observed on two-dimensional gels, is induced within 10 minutes after treatment with hydrogen peroxide (*8*). The expression

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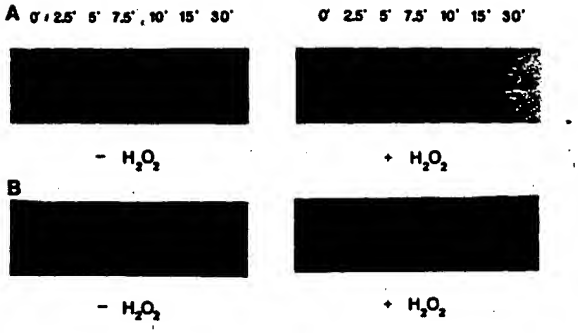

Fig. 1. Effect of hydrogen peroxide on the amount and synthesis of OxyR. (**A**) Cultures of TA4471 growing in Vogel-Bonner medium (*29*) containing 0.4 percent glucose and 0.01 percent (each) arginine, aspartate, glutamate, and methionine, were treated with hydrogen peroxide (*8, 9*). At the indicated times, 200-μl samples were centrifuged, resuspended in Laemmli buffer (*30*), and subjected to electrophoresis on 12 percent polyacrylamide gels. The proteins were transferred to nitrocellulose filters by electroblotting, and the filter was probed with a 1:2000 dilution of antibodies to an OxyR–β-galactosidase fusion protein. Bound antibody was visualized by the reaction catalyzed by alkaline phosphatase conjugated with goat antiserum to rabbit antibodies (*31*). A 1.2-kb Ssp I–Eco RV fragment of pAQ17 (*10*) carrying *oxyR* was cloned into the Sma I site of pEX2 (*32*) to generate the OxyR–β-galactosidase fusion protein of approximately 150 kD. The fusion protein was separated from other *E. coli* proteins in a cell extract on a 6 percent polyacrylamide gel. The portion of the gel containing the fusion protein was excised, and the protein was eluted from the gel fragment with an Elutrap apparatus (Schleicher & Schuell). The fusion protein (200 μg) was mixed with Freund's complete adjuvant, sonicated to form an emulsion, and injected into a New Zealand white female rabbit. The rabbit was reinjected twice (at 15-day intervals) with additional fusion protein (200 μg) mixed with Freund's incomplete adjuvant. Serum obtained 15 days after the third injection reacted predominantly with the 34-kD protein not detected in *oxyR* deletion strains. Control immunoblots showed that less than twofold differences in the amounts of OxyR could be detected. (**B**) Cells (1 ml) with and without treatment with hydrogen peroxide were labeled with 35 μCi of [^{3}H]leucine for 2-minute intervals (−2 to 0, 0.5 to 2.5, 3 to 5, 5.5 to 7.5, 8 to 10, 13 to 15, and 28 to 30). The labeling was stopped by the addition of 25 μl of 1 percent leucine, and the reactions were placed on ice. Cells were then lysed, and OxyR was precipitated with 5 μl of anti-OxyR (1:200 dilution) as described (*33*).

of an *oxyR*-regulated *katG-lacZ* fusion is also elevated within 5 minutes after treatment with hydrogen peroxide (*13*). T determine whether the increased expression of *oxyR*-regulated genes was associated with an increase in OxyR, we treated exponentially growing cells with hydrogen peroxide and examined the amount of OxyR and rate of OxyR synthesis. We first examined the amount of OxyR in an *oxyR* deletion strain carrying *oxyR* on a multicopy plasmid (TA4471, *oxyRΔ3*/pAQ17) (*14*). On this plasmid, *oxyR* is under the control of its own promoter, and the *oxyR* deletion strain carrying the plasmid shows the same adaptation to hydrogen peroxide as the corresponding wild-type strain (*10*). The TA4471 strain was grown in minimal medium and half of the culture was treated with 60 μ*M* hydrogen peroxide. Samples of the treated and untreated cells were taken immediately before the treatment and at intervals after the treatment, and were then subjected to SDS-gel electrophoresis, transferred to nitrocellulose, and probed with antibodies to an OxyR–β-galactosidase fusion protein (Fig. 1A). These immunoblots showed that there is no significant increase in the amount of OxyR after the treatment with hydrogen peroxide. Similarly, the levels of OxyR in a wild-type *E. coli* K12 strain carrying a single copy of the *oxyR* gene remained constant after treatment with hydrogen peroxide (*15*).

A culture of TA4471, with and without treatment with hydrogen peroxide, was also labeled with [^{3}H]leucine for 2-minute intervals before and after treatment with hydrogen peroxide. Portions of the cells were lysed, and the OxyR protein was precipitated with the antibody to OxyR (anti-OxyR) and subjected to electrophoresis (Fig. 1B). Whereas the synthesis of OxyR was unchanged in untreated cells, cells treated with hydrogen peroxide showed a decrease rather than an increase in OxyR synthesis during the first 10 minutes after treatment with hydrogen peroxide.

These results suggest that increased expression of the *oxyR* regulon after treatment with hydrogen peroxide is not a result of increased levels or synthesis of OxyR, but is rather a consequence of a modification of preexisting OxyR. This conclusion is in agreement with other observations about the *oxyR*-mediated response. Strains (D1210/pAQ25) (Fig. 2A) that overproduce the OxyR protein, do

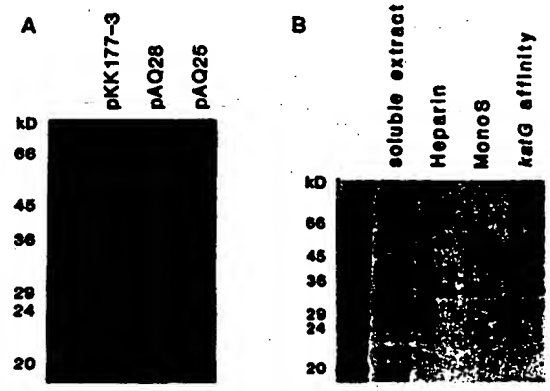

Fig. 2. Overproduction and purification of OxyR. (**A**) Samples (60 μl) of overnight cultures of D1210 (HB101/F'*lacI*^{q}) carrying pKK177-3 (*tac* promoter) (*34*), pAQ28 (*tac* promoter–*oxyR*), and pAQ25 (*tac* promoter–efficient Shine-Dalgarno–*oxyR*) were centrifuged, resuspended in Laemmli buffer, and subjected to electrophoresis on a 12 percent polyacrylamide gel. The 1.5-kb Eco RI–Hind III fragment of pAQ17 (*10*) carrying *oxyR* was cloned into the corresponding sites of pKK177-3 to generate pAQ28. To generate pAQ25, the 1.5-kb Eco RI–Eco RV fragment of pAQ17 was cloned into the Eco RI–Hinc II sites of a pUC18 derivative in which the sole Ssp I site was removed by limited Bal 31 digestion. The Eco RI–Ssp I fragment carrying the *oxyR* promoter was then replaced by annealed oligonucleotides (5'-ATTCATATTATTTCTCCTTTG-3' and 5'-AATTC-AAAGGAGAAATAATATGAAT-3') without alteration of the *oxyR* coding sequence. Finally the 1.5-kb Eco RI–Hind III fragment carrying *oxyR* with the altered Shine-Dalgarno sequence (*16*) was subcloned into pKK177-3. (**B**) Samples [0.1 μg of protein as determined by Bradford assays (*35*) with bovine serum albumin (BSA) as a standard] of the soluble starting material, peak heparin agarose, MonoS, and *katG* affinity column fractions were subjected to SDS electrophoresis and silver-stained (*36*). Whereas a few contaminating protein bands were barely visible for the MonoS fraction, no bands other than OxyR were seen for the *katG* affinity fraction. The fractions represented were treated as follows: 5 g of cells, obtained from 10 liters of D1210/pAQ25, treated with 250 mg of isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours, were resuspended in 20 ml of 50 m*M* Hepes, *p*H 7.6, 0.1 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 2 m*M* $MgCl_2$, and 50 m*M* KCl. The cells were lysed by three passages through a French pressure cell. Chromosomal DNA was then digested with DNase I, and the insoluble fraction was removed by centrifugation. The buffer concentrations of the soluble fraction were adjusted to 50 m*M* Hepes, *p*H 7.6, 0.5 m*M* EDTA, 1 m*M* DTT, 10 m*M* $MgCl_2$ (buffer Z), and 0.1*M* KCl and applied to 150 ml of heparin Sepharose CL-6B (Pharmacia) at 1 to 3 ml/min. The column was washed with 500 ml of buffer Z containing 0.1*M* KCl and 500 ml of buffer Z containing 0.2*M* KCl and then eluted with a 700-ml linear gradient of buffer Z containing 0.2 to 0.5*M* KCl. The 10-ml fraction that contained the peak of OxyR and eluted at 0.35 to 0.40*M* KCl was diluted with 40 ml of buffer Z and applied to 1 ml of Mono S HR5/5 (Pharmacia) at 0.5 ml/min. The protein was eluted with a 20-ml linear gradient of buffer Z containing KCl (0.1 to 1.0*M*). The 1-ml fraction containing the peak of OxyR was diluted with 4 ml of buffer Z containing 0.1 percent NP-40 and applied to a DNA affinity column (0.5-ml volume) prepared with oligonucleotides based on the *katG* promoter sequence (*37*) as outlined (*38*). The flowthrough was applied to the column a second time. The column was then washed with 4 ml of buffer Z containing 0.1*M* KCl and 0.1 percent NP-40, and OxyR was eluted with 2 ml of buffer Z containing 0.4*M* KCl and 0.1 percent NP-40.

not show increased expression of the *oxyR*-regulated proteins (*15*). In addition, the constitutive overproduction of *oxyR*-regulated proteins in strains carrying the *oxyR2* mutation is due to a missense mutation (Ala to Val) in OxyR rather than increased expression of the *oxyR* mRNA (*10*) or protein (*15*).

Binding of three non-homologous sequences by purified OxyR. To further characterize OxyR activation after oxidative stress, we purified the OxyR protein to homogeneity. We previously observed that OxyR negatively regulates its own expression (*10*) and found that cloning the *oxyR* promoter and open reading frame behind the well-transcribed *tac* promoter did not result in large overproduction of OxyR (Fig. 2A, pAQ28). To eliminate the site of negative autoregulation and to allow for optimal expression of the *oxyR* message, the *oxyR* promoter region (including the OxyR binding site and *oxyR* Shine-Dalgarno sequence) was replaced with an efficiently translated Shine-Dalgarno sequence (*16*) (Fig. 2A). OxyR is the predominant protein in strains carrying this construction (Fig. 2A).

Whereas a significant portion of the overproduced OxyR protein was sequestered in the insoluble fraction of the OxyR-overproducing cells, OxyR was still abundant in the soluble fraction. To avoid any complications caused by possible structural differences between the soluble and the insoluble protein, we chose to purify OxyR from only the soluble fraction. Full purification (no other bands were visible on a silver-stained gel loaded with 0.5 μg of protein) was achieved by fractionation on three columns (Fig. 2B). Fractionation on a heparin-agarose column eliminated most of the cellular proteins. A second fractionation step on MonoS resin removed additional contaminants and resulted in concentration of the OxyR protein. The remaining contaminants were eliminated on a sequence-specific DNA affinity matrix consisting of ligated oligonucleotides containing the OxyR-binding site upstream of *katG* (portions of the *katG* affinity fraction shown in Fig. 2B were used in all subsequent experiments).

Crude extracts from OxyR-overproducing cells protect sequences upstream of the *katG*, *ahpC*, and *oxyR* genes from DNase I digestion that do not show any apparent sequence similarity (*17*). To determine whether OxyR purified to homogeneity on the basis of binding to the *katG* sequence could still bind the *ahpC* and *oxyR* sequences, we incubated samples of the fraction shown in Fig. 2B with *katG*, *ahpC*, and *oxyR* promoter fragments and assayed for the ability to protect against DNase I digestion. Samples containing less than 5 ng of purified OxyR were able to fully protect all of the promoter fragments against DNase I digestion (*18*) (Fig. 5). These results showed that purified OxyR is capable of binding to the three different sequences at the *katG*, *ahpC*, and *oxyR* promoters. The sites bound by OxyR are quite large (>45 nucleotides) suggesting that OxyR might bind as a multimer, but no obvious inverted or tandem repeats are seen within the protected regions.

OxyR activates transcription on oxidation. To investigate the

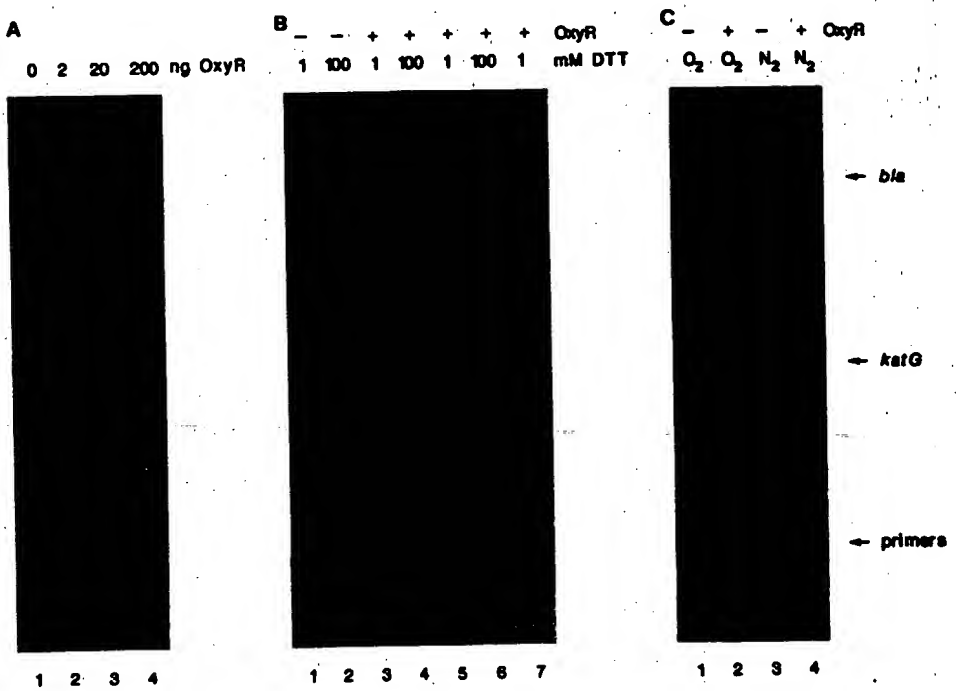

Fig. 3. Activation of *katG* expression in vitro by oxidized but not reduced OxyR. (**A**) Increasing amounts [2, 20, and 200 ng of protein based on Bradford assays (*35*) before the centrifugation step below] of purified OxyR were added to RNA polymerase and a plasmid (pBT22) (*19*) carrying the *katG* and *bla* genes, and the resulting in vitro transcription products were examined by primer extension. The transcription assays were carried out as follows: A sample (50 μl) of purified OxyR with carrier BSA (15 μg) was centrifuged through 800 μl of Sephadex (Boehringer Mannheim) into the transcription buffer (40 m*M* tris-Cl, pH 7.9, 0.1*M* KCl, 10 m*M* $MgCl_2$) containing 1 m*M* DTT, 5 percent glycerol, and 0.1 percent NP-40. A portion (5 μl) of centrifuged OxyR together with 2.5 μl of H_2O was then incubated with pBT22 DNA (0.2 μg in 36.5 μl of transcription buffer) for 10 minutes at 37°C. RNA polymerase holoenzyme [0.5 μg (*39*) in 5 μl of transcription buffer] was added to the previously bound template, and the reaction was incubated for an additional 10 minutes at 37°C. After the addition of 1 μl of a 25 m*M* NTP mixture, the reactions were incubated for 5 minutes at 37°C. The reactions were terminated by the addition of phenol and extracted several times with phenol and chloroform. Then a labeled primer was added and extended with reverse transcriptase (*10, 15*). (**B**) OxyR was assayed for the ability to activate *katG* transcription in the presence of 1 or 100 m*M* DTT. The OxyR fractions represented in lanes 3 through 7 were treated as follows. Purified OxyR (50 μl) with BSA was centrifuged into transcription buffer containing 1 m*M* DTT, glycerol, and NP-40 as described above. After a sample was removed (lane 3), the DTT concentration was adjusted to 100 m*M*. Another sample was removed (lane 4) before the remainder was centrifuged through a second Sephadex column equilibrated with transcription buffer containing 1 m*M* DTT, glycerol, and NP-40. After a third sample (lane 5) was removed OxyR was again treated with 100 m*M* DTT (lane 6) and subsequently centrifuged into 1 m*M* DTT (lane 7). The OxyR samples [1 μl (lane 3), 1.5 μl (lane 4), 3 μl (lane 5), 3.5 μl (lane 6), and 5 μl (lane 7), to compensate for dilution by the added DTT or for loss during centrifugation] were then assayed for transcription as described for (A). The DTT concentrations of the transcription reactions corresponding to lanes 2, 4, and 6 were adjusted to 100 m*M* by the addition of 2.5 μl of 1.7*M* DTT instead of 2.5 μl of H_2O. (**C**) OxyR (1 μl of the centrifuged sample described for (A) was assayed for the ability to activate *katG* transcription in the presence of air- or nitrogen-saturated buffers. The transcription assays for lanes 1 and 2 were performed as described for (A) except that the DTT concentration of the reaction mixture was adjusted to 10 m*M* by the addition of 2.5 μl of 0.2*M* DTT instead of 2.5 μl of H_2O. The transcription assays for lanes 3 and 4 were performed (as described for lanes 1 and 2) in the Anaerobic Facility (containing less than 5 ppm of O_2) in the Laboratory of Biochemistry at the National Heart Lung and Blood Institute.

mechanism by which OxyR regulates the expression of the *oxyR* regulon, we first assayed the ability of extracts from OxyR-overproducing cells to activate expression of the *ahp* genes in an in vitro transcription-translation assay. The addition of extracts prepared by sonication stimulated the expression of the *ahp* genes, but not a control *bla* gene (encoding β-lactamase) (*18*). Initially this finding was surprising because neither the cells nor the extracts had been treated with hydrogen peroxide. Suspecting that OxyR could be activated solely on release from the reducing environment of the cell into air-saturated buffers [even buffers containing 1 m*M* dithiothreitol (DTT)], we prepared extracts in the presence of various antioxidants and reductants. Extracts from the OxyR-overproducing strain prepared and assayed in the presence of 100 m*M* DTT no longer activated the *ahp* genes in the transcription-translation assay (*18*). The high concentration of DTT did not alter the basal levels of *ahp* or *bla* expression, and the inactive extracts could activate expression if the 100 m*M* DTT was removed by dialysis (*18*). Concentrations of DTT as low as 10 m*M* prevented the activation if extracts were prepared and assayed under semianaerobic conditions (tubes purged with argon) (*18*). These results suggested that the oxidation state of the OxyR-enriched extracts affected their ability to stimulate expression of the *ahp* genes in vitro.

To determine whether OxyR was the sole sensor of oxidation and the only component required to activate *oxyR*-regulated gene expression, we examined the ability of purified OxyR to regulate expression of *oxyR*-regulated genes in more defined transcription assays. Samples of the most highly purified OxyR fraction were incubated with RNA polymerase holoenzyme, and plasmids carrying the *oxyR*-regulated *ahpC* (pAQ27) (*13*) or *katG* genes (pBT22) (*19*) as well as the *bla* gene as a control. The transcription products generated in vitro were then examined by primer extension assays. Increasing amounts of OxyR increased the transcription of both the *ahpC* (*15*) and *katG* genes (Fig. 3A) while having no effect on the expression of the *bla* gene. Densitometer scans of Fig. 3A showed that the addition of OxyR caused an induction of the *katG* message of more than 100-fold. The start sites of the transcripts generated in vitro, as determined by the lengths of the primer extension products, agree with the transcription starts seen for the *ahpC* and *katG* genes in vivo (*13*) and described for the *bla* gene (*20*).

We also assayed the ability of purified OxyR to activate transcription in the presence of 1 and 100 m*M* DTT to determine whether the purified protein showed the same sensitivity to oxidation and reduction as seen for the cell extracts. The high concentration (100 m*M*) of DTT did not reduce transcription in general since neither *bla* expression nor the basal level of *katG* expression was affected (Fig. 3B, lane 1 compared to lane 2). However, OxyR incubated in the presence of 100 m*M* DTT no longer activated expression of *katG* (Fig. 3B, lane 3 compared to lane 4). The inactivation of OxyR by 100 m*M* DTT was readily reversible since OxyR regained the ability to activate *katG* expression upon removal of the high concentration of DTT by gel filtration (Fig. 3B, lane 5). We also found that the same OxyR sample could then be reinactivated by 100 m*M* DTT and reactivated by removal of the DTT for a second time (Fig. 3B, lanes 6 and 7). These results suggest that the ability of OxyR to activate transcription is sensitive to the oxidation state of OxyR. The interconversion of OxyR between the active and inactive forms is also readily reversible within the 5-minute period required to remove the DTT.

The finding that DTT, a reductant, could reversibly inactivate the OxyR protein suggested that the redox state of the OxyR protein was coupled to its ability to stimulate transcription. However, the high concentrations of DTT might inhibit activation by denaturing OxyR or by chelating a metal required for function. To distinguish between these possibilities, we assayed the ability of OxyR to activate transcription under anaerobic conditions. In air-saturated buffers, OxyR is active in the presence of 10 m*M* DTT (Fig. 3C, lanes 1 and 2). If the transcription assays were performed in the presence of 10 m*M* DTT under a nitrogen atmosphere, OxyR no longer activated *katG* expression (Fig. 3C, lanes 3 and 4). These parallel assays (in which the only variable is the presence or absence of oxygen) strengthen our conclusion that the ability of OxyR to activate transcription is directly dependent on the state of OxyR oxidation.

DNA-binding of oxidized and reduced OxyR is distinct. Since we previously found that OxyR negatively autoregulates its own expression in vivo (*10*), we also examined the ability of OxyR to regulate *oxyR* transcription under oxidizing (1 m*M* DTT) and reducing (100 m*M* DTT) conditions with purified OxyR and RNA polymerase in vitro. The start of the *oxyR* transcript generated in vitro is consistent with the transcription start seen in vivo (*10*), and high levels of *oxyR* transcription are seen in the absence of OxyR (Fig. 4, lanes 1 and 3). The *oxyR* expression was reduced substantially in the presence of both reduced and oxidized OxyR (Fig. 4, lanes 2 and 4). The oxidized form of OxyR appears to be more efficient than the reduced form in repressing its own expression, in agreement with the observation that the synthesis of OxyR is decreased in cells treated with hydrogen peroxide compared to untreated cells (Fig. 1B).

We consistently see a decrease in the synthesis of the control *bla* gene in transcription reactions containing both the oxidized OxyR protein and the *oxyR* gene (Fig. 4, lane 2). This decreased expression of the *bla* gene is likely to be due to the titration of a limiting component in our transcription assays by the extremely high expression of a small RNA that is encoded in an orientation opposite to the *oxyR* gene and whose expression is highly induced by hydrogen peroxide in vivo (*21*). Repression by oxidized OxyR without a decrease in *bla* expression was observed in coupled transcription-translation assays in which all components are in greater excess (*18*).

The observation that reduced OxyR could act as a repressor suggested that OxyR was capable of binding to DNA under both oxidizing and reducing conditions. To investigate this possibility and further characterize the reduced form of OxyR, we assayed the ability of OxyR to bind to the *katG*, *ahpC*, and *oxyR* promoters

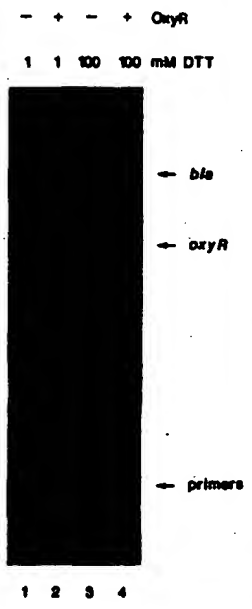

Fig. 4. Both oxidized and reduced OxyR repress *oxyR* expression in vitro. OxyR (1 μl of the centrifuged sample described in Fig. 3A) was assayed for the ability to repress transcription of the *oxyR* gene in the presence or absence of 100 m*M* DTT as described in Fig. 3B. A pUC12 plasmid (0.2 μg) carrying *oxyR* (pAQ17) (*10*) was used as the template. A distinct *bla* transcript extension product was visible on long exposures of lane 2, while no *oxyR* transcript extension product was detected.

under both conditions. DNase I assays in the presence of 1 and 100 m*M* DTT clearly show that OxyR binds to all three promoters, with high affinity under both oxidizing and reducing conditions (Fig. 5). Therefore, the conversion of reduced OxyR to a transcriptional activator upon oxidation is not likely due to significantly increased DNA-binding by the oxidized form of OxyR, but is probably due to a conformational change in OxyR already bound to the promoter regions.

In support of this conclusion, the footprints of oxidized and reduced OxyR are different. A distinct DNase I hypersensitive site (indicated by arrows in Fig. 5) is seen in the footprints with reduced-inactive but not oxidized-active OxyR. A DNase I hypersensitive site was also previously seen for IlvY (a member of the family of regulators showing homology to OxyR) in the absence but not in the presence of the inducer of the IlvY-mediated response (*22*). The lengths of the OxyR footprints at the *katG* and *oxyR* promoters are also different under oxidizing and reducing conditions (Fig. 5). While the footprint at the *katG* promoter is shortened in 100 m*M* DTT, the footprint at the *oxyR* promoter shows a clear extension under the reducing conditions. These observations suggest that the OxyR protein may change its contacts with the DNA. Although such alterations in footprint pattern are rare, *E. coli* RNA polymerase has also been found to drastically alter its contacts with DNA as the enzyme undergoes the transition from a closed to an open complex (*23*). The altered footprint of reduced OxyR is not an artifact due to the high concentrations of DTT since a noninducible OxyR mutant protein (resulting from the conversion of the cysteine residue at position 199 to serine), which no longer functions as an activator in vivo, shows a DNase I protection pattern in 1 m*M* DTT that is identical to the pattern seen for the wild-type protein in 100 m*M* DTT (*15*).

Oxidation as a mechanism for regulation. Our results show that OxyR, a transcriptional activator of genes induced by hydrogen peroxide, is activated directly by oxidation and is therefore both the sensor and transducer of an oxidative stress signal. We postulate that low amounts of reduced OxyR molecules are present in the cells at all times, bound to the promoters of *oxyR*-regulated genes. These OxyR molecules are poised to be activated by low amounts of hydrogen peroxide which presumably enter the cell by diffusion. Such a system allows for efficient and rapid induction of defense genes. Some of the other bacterial regulators that show homology to OxyR have also been found to be bound to the promoters that they regulate in both the presence and absence of inducer (*22, 24*) suggesting that members of this family of regulators may all be ideally designed for responses requiring immediate induction.

The differences in the footprints between reduced and oxidized OxyR at the *katG*, *ahpC*, and *oxyR* promoters suggest that a distinct conformational change in the OxyR protein is associated with the transition from the reduced (inactive) to the oxidized (active) state. Possibly the conformational change exposes an activation domain as has been proposed for numerous eukaryotic activators (*25*). Alternatively the conformational change may be associated with a change in oligomerization. Leucine residues that are spaced at intervals of seven amino acids reminiscent of a leucine zipper dimerization domain are highly conserved among the LysR-OxyR family of regulators (*15*). The details of how transcriptional activators contact polymerase to activate transcription are still largely unknown in both prokaryotes and eukaryotes. Hence, structural and genetic studies of the differences between the oxidized and reduced forms of OxyR should provide important insights into transcriptional activation in general.

The finding that the interactions of reduced OxyR with the individual promoters are different is also intriguing. Perhaps the different sequences at the three promoters dictate different conformational changes and roles for OxyR at the three promoters. At the *oxyR* promoter, OxyR activates transcription in one direction and represses transcription in the other, whereas OxyR probably only acts to induce transcription at the *katG* and *ahpC* promoters. Bölker and Kahmann have also characterized the binding of OxyR to the phage Mu *mom* promoter, which is repressed by OxyR (*11*). Interestingly, at this promoter, OxyR discriminates between methylated and unmethylated DNA (*11*). Further analysis of the specific DNA contacts made by reduced and oxidized OxyR at the different promoters should elucidate how OxyR is interacting with the different sequences and how a single protein can recognize several sequences that do not show apparent homology.

The nature of the oxygen species causing the conformational change and activation of OxyR in our assays has not yet been identified. Since OxyR is activated simply by the presence of air in 10 m*M* DTT in our in vitro assays, we suggest that reactive oxygen species are generated by the dissolved oxygen in our purification and assay buffers. Air-saturated solutions containing low concentrations of thiol compounds or detergents, both present in our buffers, are known to contain reactive oxygen species (*26*). We have prevented OxyR activation on removal of the 100 m*M* DTT by the addition of catalase (which breaks down hydrogen peroxide), suggesting that concentrations of hydrogen peroxide capable of activating OxyR are present under the aerobic conditions (*15*). Presumably, the rate of OxyR reduction by 100 m*M* DTT, but not 10 m*M* DTT, is sufficient to inhibit OxyR activation by the low concentrations of oxidants present in our buffer solutions.

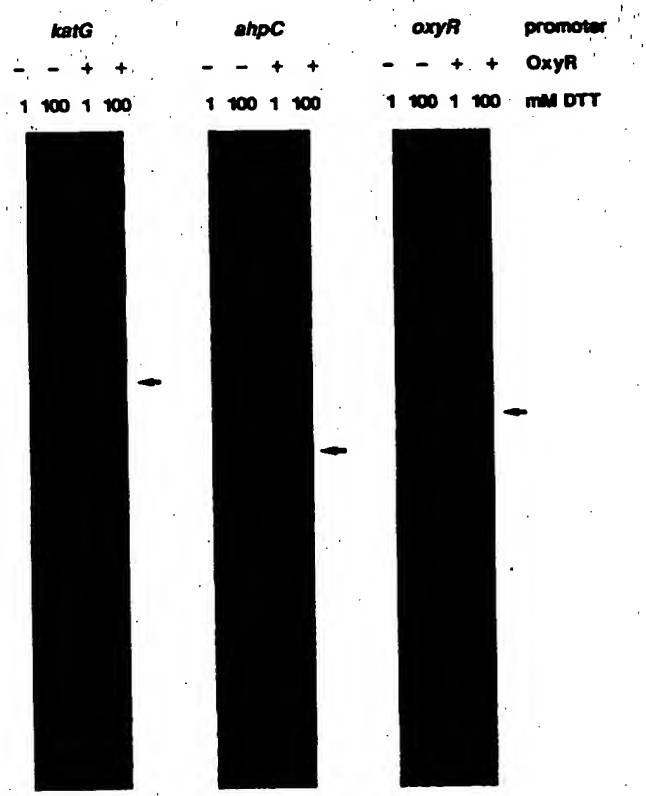

Fig. 5. Binding of oxidized and reduced OxyR to the *katG*, *ahpC*, and *oxyR* promoters. Samples of purified OxyR were incubated with *katG* (30 ng) (*13*), *ahpC* (30 ng) (*13*), and *oxyR* (7.5 ng) (*10*) promoter fragments in the presence of 1 and 100 m*M* DTT for 10 minutes. Subsequently, DNase I was added, and the samples were analyzed as described (*10, 13*). High concentrations of DTT do not alter the DNase I digestion patterns in the absence of OxyR.

Within the cell, OxyR is only a transcriptional activator for a limited time after oxidative stress since pulse-labeling experiments have shown that the induction of *oxyR*-regulated genes is predominant only during the first 10 minutes after treatment with hydrogen peroxide (*8*). However, the total levels of OxyR do not decrease significantly at any time point after treatment with the oxidant. Pulse-chase experiments also showed that the stability of the OxyR protein does not change after treatment with hydrogen peroxide (*15*). These observations suggest that bacterial cells possess a mechanism which allows for the inactivation of OxyR after a defined period of time, possibly by re-reduction of OxyR after the hydrogen peroxide is removed by catalase.

The nature of the redox-active center in OxyR is of interest. The candidates for this redox-active center are limited since the oxidation and reduction is readily reversible. Inter- or intra-molecular disulfide bonds meet this criterion, but we have never observed significant levels of a form of OxyR larger than 34 kD on immunoblots of non-reducing SDS gels probed with the OxyR antibody suggesting that no intermolecular disulfide bonds are formed (*18*). In addition, a mutant form of OxyR in which five of the six cysteine residues have been converted to serine shows wild-type induction of *oxyR*-regulated genes in vivo (*15*). It is also unlikely that OxyR is activated by the formation of an intermolecular disulfide bond with a small molecule, such as glutathione, since OxyR is activated by gel filtration that separates the protein from all small molecules in our assays. A cofactor or metal associated with OxyR might also be sensitive to oxidation; however, the addition of chelators did not effect the ability of OxyR to activate transcription (*15*). The one essential cysteine may also be reversibly oxidized to a sulfenic acid. Alternatively, OxyR may be activated by direct binding of the reactive oxygen species. Additional physical studies of OxyR should provide insights into how a protein can be oxidized in a very specific fashion.

The activities of several metabolic enzymes (*27*) and one other regulator of gene expression, the iron-responsive element (IRE) binding protein, have been found to be sensitive to oxidation in vitro (*28*). The IRE binding protein, a regulator of ferritin expression in human cells, requires free sulfhydryl groups for binding to IREs. This finding suggests that cells sense the presence or absence of iron, by modulating the reduction or oxidation of the IRE binding protein. It is likely that the activities of other regulators of gene expression are modulated by oxidation and reduction. OxyR may play a role in protecting the bacterial cell against the oxidative burst which is released when the bacterial cells encounter macrophages. Other prokaryotic and eukaryotic regulators may be activated by this oxidative burst or by the oxidants associated with degenerative diseases. Eye and lung tissue which are in contact with air may provide sources for identifying additional examples of redox-sensitive regulators. Regulators in chloroplasts and mitochondria, the sites of electron transport associated with photosynthesis and oxidative phosphorylation, may also be sensitive to oxidation or reduction, and the redox state of additional regulators may be coupled to the presence or absence of metals. Whether oxidation-reduction is a general mechanism of regulation remains to be determined as additional signal transduction pathways are characterized.

REFERENCES AND NOTES

1. T. V. O'Halloran, B. Frantz, M. K. Shin, D. M. Ralston, J. G. Wright, *Cell* 56, 119 (1989); L. M. Shewchuk, G. L. Verdine, C. T. Walsh, *Biochemistry* 28, 2331 (1989); L. M. Shewchuck *et al.*, *ibid.*, p. 2340; J. D. Helmann, Y. Wang, I. Mahler, C. T. Walsh, *J. Bacteriol.* 171, 222 (1989).
2. I. Teo, B. Sedgwick, M. W. Kilpatrick, T. V. McCarthy, T. Lindahl, *Cell* 45, 315 (1986).
3. A. D. Grossman, D. B. Straus, W. A. Walter, C. A. Gross, *Genes Dev.* 1, 179 (1987); D. B. Straus, W. A. Walter, C. A. Gross, *Nature* 329, 348 (1987).
4. P. K. Sorger, M. J. Lewis, H. R. B. Pelham, *Nature* 329, 81 (1987); P. K. Sorger and H. R. B. Pelham, *Cell* 54, 855 (1988).
5. B. N. Ames, *Science* 221, 1256 (1983); B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine* (Clarendon, Oxford, 1985); P. A. Cerutti, *Science* 227, 375 (1985); H. Sies, *Angew. Chem. Int. Ed. Engl.* 25, 1058 (1986); C. E. Cross *et al.*, *Ann. Intern. Med.* 107, 526 (1987).
6. J. A. Imlay and S. Linn, *J. Bacteriol.* 169, 2967 (1987); L. K. B. Walkup and T. Kogoma, *ibid.* 171, 1476 (1989); J. T. Greenberg and B. Demple, *ibid.*, p. 3933; E. Chan and B. Weiss, *Proc. Natl. Acad. Sci. U.S.A.* 84, 3189 (1987); S. M. Keyse and R. M. Tyrrell, *ibid.* 86, 99 (1989); I. H. Kim, K. Kim, S. G. Rhee, *ibid.*, p. 6018; S. Wolff, J. K. Wiencke, V. Afzal, J. Youngblom, F. Cortes, in *Low Dose Radiation: Biological Basis of Risk Assessment*, K. F. Baverstock and J. W. Stather, Eds. (Taylor and Francis, London, 1989), pp. 446–454.
7. B. Demple and J. Halbrook, *Nature* 304, 466 (1983).
8. M. F. Christman, R. W. Morgan, F. S. Jacobson, B. N. Ames, *Cell* 41, 753 (1985).
9. R. W. Morgan, M. F. Christman, F. S. Jacobson, G. Storz, B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* 83, 8059 (1986).
10. M. F. Christman, G. Storz, B. N. Ames, *ibid.* 86, 3484 (1989).
11. M. Bölker and R. Kahmann, *EMBO J.* 8, 2403 (1989).
12. K. Tao, K. Makino, S. Yonei, A. Nakata, H. Shinagawa, *Mol. Gen. Genet.* 218, 371 (1989).
13. L. A. Tartaglia, G. Storz, B. N. Ames, *J. Mol. Biol.* 210, 709 (1989).
14. The multicopy plasmid was used to amplify the OxyR signal slightly.
15. G. Storz, L. A. Tartaglia, B. N. Ames, unpublished results.
16. L. Gold and G. Stormo, *Methods Enzymol.*, in press.
17. Sequences bound by OxyR at the following promoters (*13*)

    katG 5'-ATGTAAGATCTCAACTATCGCATCCGTGGATTAATTCAATTATAA-3'

    ahpC 5'-GGGTTGTTAGTTAACGCTTATTGATTTGATAATGGAAACGCATTAG-3'

    oxyR 5'-GCCACGATAGTTCATGGCGATAGGTAGAATAGCAATGAACGATTA-3'
18. L. A. Tartaglia, G. Storz, B. N. Ames, unpublished results.
19. B. L. Triggs-Raine and P. C. Loewen, *Gene* 52, 121 (1987).
20. J. Brosius, R. L. Cate, A. P. Perlmutter, *J. Biol. Chem.* 257, 9205 (1982).
21. G. Storz and B. N. Ames, unpublished results.
22. R. C. Wek and G. W. Hatfield, *J. Mol. Biol.* 203, 643 (1988).
23. B. Krummel and M. J. Chamberlin, *Biochemistry* 28, 7829 (1989).
24. R. F. Fisher and S. R. Long, *J. Bacteriol.* 171, 5492 (1989); S. Lindquist, F. Lindberg, S. Normark, *ibid.*, p. 3746; M. A. Schell and E. F. Poser, *ibid.*, p. 837; M. L. Urbanowski and G. V. Stauffer, *ibid.*, p. 5620; J. Ostrowski and N. M. Kredich, *ibid.* 172, 779 (1990); R. K. Rothmel *et al.*, *ibid.*, p. 922.
25. M. Ptashne, *Nature* 335, 683 (1988); P. J. Mitchell and R. Tjian, *Science* 245, 371 (1989).
26. K. D. Held and D. C. Melder, *Rad. Res.* 112, 544 (1987); M. Lever, *Anal. Biochem.* 83, 274 (1977).
27. D. M. Ziegler, *Annu. Rev. Biochem.* 54, 305 (1985); B. B. Buchanan, *Annu. Rev. Plant Physiol.* 31, 341 (1980).
28. M. W. Hentze, T. A. Rouault, J. B. Harford, R. D. Klausner, *Science* 244, 357 (1989).
29. H. J. Vogel and D. M. Bonner, *J. Biol. Chem.* 218, 97 (1956).
30. U. K. Laemmli, *Nature* 227, 680 (1970).
31. M. S. Blake, K. H. Johnston, G. J. Russell-Jones, E. C. Gotschlich, *Anal. Biochem.* 136, 175 (1984); D. A. Knecht and R. L. Dimond, *ibid.*, p. 180.
32. K. K. Stanley and J. P. Luzio, *EMBO J.* 3, 1429 (1984).
33. G. Storz *et al.*, *J. Bacteriol.* 171, 2049 (1989).
34. Derived from pKK223-3, J. Brosius and A. Holy, *Proc. Natl. Acad. Sci. U.S.A.* 81, 6929 (1984).
35. M. M. Bradford, *Anal. Biochem.* 72, 248 (1976).
36. H. Blum, H. Beier, H. J. Gross, *Electrophoresis* 8, 93 (1987).
37. 5'-AATTGAATTAATCCACGGATGCGATAGTTGAGATCTTGATC-3' and

    5'-AAGATCTCAACTATCGCATCCGTGGATTAATTCAATTGATC-3'.
38. J. T. Kadonaga and R. Tjian, *Proc. Natl. Acad. Sci. U.S.A.* 83, 5889 (1986).
39. The RNA polymerase was a gift of the M. Chamberlin laboratory.

40. We thank S. Adhya for supporting experiments that were conducted by G.S. in the Laboratory of Molecular Biology at the National Cancer Institute, C. Wu, S. Bell, J. Thorner, and S. Garges for advice and assistance with techniques, members of the Chamberlin laboratory for the advice and the gift of RNA polymerase holoenzyme, and T. Stadtman, D. Grahame, and M. Atkey for use of and assistance with the Anaerobic Facility at the National Institutes of Health (NIH). Supported by NIH grant GM 19993 (B.N.A.), National Cancer Institute Outstanding Investigator grant CA39910 (B.N.A.), National Institute of Environmental Health Sciences (NIEHS) Center grant ES01896, NIH training grant GM07232 (G.S.), a fellowship from the National Cancer Institute's Biotechnology Training Program (G.S.), and NIEHS training grant ES07075 (L.A.T.)

6 December 1989; accepted 12 March 1990

JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1275–1284
0021-9193/95/$04.00+0

Vol. 177, No. 5

Mutational Analysis of the Redox-Sensitive Transcriptional Regulator OxyR: Regions Important for Oxidation and Transcriptional Activation

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Received 26 July 1994/Accepted 15 December 1994

OxyR is a redox-sensitive transcriptional regulator of the LysR family which activates the expression of genes important for the defense against hydrogen peroxide in *Escherichia coli* and *Salmonella typhimurium*. OxyR is sensitive to oxidation and reduction, and only oxidized OxyR is able to activate transcription of its target genes. Using site-directed mutagenesis, we found that one cysteine residue (C-199) is critical for the redox sensitivity of OxyR, and a C-199→S mutation appears to lock the OxyR protein in the reduced form. We also used a random mutagenesis approach to isolate eight constitutively active mutants. All of the mutations are located in the C-terminal half of the protein, and four of the mutations map near the critical C-199 residue. In vivo as well as in vitro transcription experiments showed that the constitutive mutant proteins were able to activate transcription under both oxidizing and reducing conditions, and DNase I footprints showed that this activation is due to the ability of the mutant proteins to induce cooperative binding of RNA polymerase. Unexpectedly, RNA polymerase was also found to reciprocally affect OxyR binding.

The OxyR protein is a transcriptional activator of genes important for the defense against oxidative stress in *Escherichia coli* and *Salmonella typhimurium* (14, 37). Upon exposure to hydrogen peroxide, OxyR induces the expression of several genes, including *katG* (encoding HPI catalase), *ahpCF* (encoding an alkyl hydroperoxide reductase), *dps* (encoding a nonspecific DNA-binding protein), *gorA* (encoding glutathione reductase), and *oxyS* (encoding a small untranslated, regulatory RNA), and the cells become more resistant to oxidative stress (3, 4, 39, 41). During normal growth, OxyR also acts as a repressor, negatively autoregulating its own expression and the expression of the Mu phage *mom* gene (6, 14, 38). Here and in the accompanying paper (20), we identify regions of the OxyR protein critical for activation and repression.

OxyR is 34 kDa in size and belongs to the LysR-type family of transcriptional regulators (6, 14, 37, 43). LysR family members are all DNA-binding proteins which positively regulate expression of their target genes, and many of the regulators also repress their own expression (reviewed in reference 29). The ability of most LysR-type proteins to activate transcription is dependent on the presence of a coinducer, such as octopine for the *Agrobacterium tumefaciens* OccR protein (42), indoleglycerol phosphate for *Pseudomonas aeruginosa* TrpI (12), *N*-acetylserine for *E. coli* CysB (26), and flavonoids for the NodD proteins of different species of *Rhizobium* (reviewed in reference 16). The central region of the LysR-type proteins is assumed to be involved in coinducer binding, since *Rhizobium leguminosarum* NodD and *Pseudomonas putida* NahR mutations which caused an altered response to the inducer mapped to the central domain (9, 18, 24, 30), and an exchange between the central domains of NodD proteins from different *Rhizobium* species leads to a different coinducer specificity of the resulting hybrid proteins (31). The fact that members of the LysR-type family of transcriptional regulators have only little homology in this protein region probably reflects the variety of coinducers to which these proteins respond. Unlike many of the other LysR-type regulators, the OxyR protein does not appear to bind a coinducer but rather is activated by direct oxidation. In vitro transcription experiments with purified components showed that the oxidized but not the reduced form of OxyR was able to activate transcription (35), but the redox-active center has not been characterized.

A transcriptional activator may increase RNA polymerase binding, open complex formation, or promoter clearance (reviewed in reference 2). A few studies suggest that LysR-type regulators contact the α subunit of RNA polymerase and act to increase polymerase binding to the promoter. OxyR binds adjacent to the −35 sequence of the positively regulated promoters, and Tao et al. have shown that OxyR acts cooperatively to increase RNA polymerase binding to the *katG* promoter (36). Mutations in the α subunit of polymerase, such as a change of R-265 to C (R265C), prevented activation by OxyR (36). Similarly, mutations at codon 271 of the α subunit of RNA polymerase prevent activation by the LysR-type regulator CysB (40). The domains in OxyR and other LysR family members that touch RNA polymerase have not been identified.

In recent studies on the DNA binding properties of OxyR, we found that the reduced and oxidized (activated) forms of OxyR require different DNA contacts for binding (41). Oxidized OxyR binds in four adjacent major grooves, while reduced OxyR contacts two pairs of adjacent major grooves separated by one helical turn. The reduced protein has significantly reduced affinities for the *katG* and *ahpCF* promoters, showing that only a subset of the OxyR-regulated promoters

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carry determinants for the binding f reduced OxyR. The two binding modes probably allow OxyR to repress the *oxyR* and *mom* promoters during normal growth, while activating *katG*, *ahpCF*, *dps*, *gorA*, and *oxyS* only upon oxidative stress. These studies also suggest that the conformations of reduced and oxidized OxyR are significantly different.

In the present study, we used site-directed as well as random mutagenesis to characterize the domains of the OxyR protein required to sense oxidative stress and affect transcription initiation. The six possible redox-reactive cysteines in OxyR were mutagenized to serine, and a single cysteine (Cys-199) was found to be critical for activity. Random mutagenesis revealed that a region around this critical cysteine is involved in transcriptional activation, since several mutations causing the constitutive phenotype mapped to this region. We were also able to show that the ability of mutants to activate transcription correlates with their ability to induce RNA polymerase binding. Moreover, DNase I footprinting assays demonstrated that while OxyR induces polymerase binding, RNA polymerase also reciprocally influences OxyR binding.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are described in Table 1. Strain GSO5 was constructed as follows. The 0.2-kb *Hin*dIII-*Ssp*I fragment of pAQ17 (14), carrying the *oxyR-oxyS* promoter region, was cloned into the unique *Sma*I site of pTS7 (1) to create the *galK* fusion. The plasmid was then recombined onto λ_{Y2055} (cI857 *galK nin5*) and integrated into the *att*λ site of SA2692 (1). A Δ*oxyR::kan* deletion-insertion mutation (3) was subsequently moved into the strain by P1 transduction. pAQ5 (34) was used for the random mutagenesis and is a pACYC184 derivative which carries *oxyR* on a *Bam*HI-*Eco*RV fragment. Since these pACYC184-derived plasmids did not prove to be suitable for double-strand sequencing, the *Bam*HI-*Hin*dIII *oxyR* fragments were subcloned into M13mp18 for sequencing. For overproduction of the mutant OxyR proteins, *oxyR* was cloned behind the P_{tac} promoter of pKK177-3 as follows. First, the *Ssp*I-*Hin*dIII fragment of wild-type *oxyR* in pUC18 carrying the modified Shine-Dalgarno sequence (35) was replaced with the same fragment of the mutant *oxyR* genes from pACYC184. These constructs were sequenced, and subsequently the *Eco*RI-*Hin*dIII fragment was subcloned into pKK177-3. All sequencing and subcloning were carried out by standard procedures.

Media and growth conditions. Strains were grown in LB medium (25), and ampicillin (100 μg/ml [final concentration]), kanamycin (25 μg/ml), chloramphenicol (25 μg/ml), or tetracycline (15 μg/ml) was added when appropriate. For overproduction of the mutant proteins, the cells were grown in TB medium (28). The resistance of strains to hydrogen peroxide and cumene hydroperoxide was assayed by zones of inhibition, which were determined as described previously (13) except that the strains were grown in and plated on LB medium containing the appropriate antibiotics.

Mutagenesis. Site-directed mutagenesis was carried out with an oligonucleotide-directed in vitro mutagenesis system (Amersham, Arlington Heights, Ill.). The base pair substitutions that were introduced are listed in Table 2. Random mutagenesis with hydroxylamine was carried out with approximately 10 μg of purified pAQ5 plasmid DNA as described previously (15). The DNA (20 μl) was mixed with 100 μl of a 0.5 M potassium phosphate–5 mM EDTA solution (pH 6) and 80 μl of a freshly prepared 1 M hydroxylamine solution (pH 6) and incubated for 60 min at 65°C. The samples were dialyzed extensively against Tris-EDTA buffer and used directly (5- to 10-μl aliquots) to transform *E. coli* XL1-blue cells. The XL1-blue transformants were rinsed off the plates, and the plasmid DNA was reisolated and then used to transform the GSO5 recipient strain. Finally, the GSO5 transformants were screened for the desired phenotype on MacConkey agar plates.

Protein overexpression and purification. For overproduction of the mutant OxyR proteins, *oxyR* deletion strains (TA4484) with the corresponding pKK177-3 derivatives were grown to mid-exponential phase and then induced with 250 μg of IPTG (isopropyl-β-D-thiogalactopyranoside) per ml for 2 h. The cells were lysed by several passages through a French pressure cell, and the insoluble fraction was removed by centrifugation. The soluble fraction was then applied to a heparin-Sepharose column (Pharmacia, Piscataway, N.J.). The purification procedure was carried out as described previously (35) except that the buffer Z contained 50 mM HEPES (*N*-2-hydroxypiperazine-*N*′-2-ethanesulfonic acid) (pH 8), 5 mM $MgCl_2$, 0.5 mM EDTA (pH 8), and 10% (vol/vol) glycerol. The A233V and E225K mutants were eluted with a 0.1 to 0.4 M KCl gradient instead of the second wash with buffer Z containing 0.2 M KCl. Both the A233V and E225K mutants eluted at about 0.2 M KCl. Aliquots of the peak fractions were analyzed on sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) gels, using the buffer system of Laemmli (21). The protein concentration in all of the preparations was approximately 0.8 mg/ml, and roughly 8 mg of approximately 90% pure OxyR protein was obtained from a 1-liter cell culture.

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
XL1-blue	F′ [*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)]	8
D1210	HB101/F′ [*lacI*^{q}]	5
TA4112	*oxyR*Δ*3*	13
TA4484	*oxyR*Δ*3*, pMC7	39
SA2692	HB101 *recA*$^+$ Δ*lac* Δ*gal-165*	1
GSO5	SA2692 Δ*oxyR::kan* (λ_{Y2055} *oxyS-galK*)	This study
Plasmids		
pTS7	pBR322 *int*$^+$ P′P; promoterless *lacZ* and *galETK*, Ap^r	1
pMC7	*lacI*^{q} Tc^r	10
pACYC184	Cm^r Tc^r	11
pKK177-3	P_{tac} promoter, derivative of pKK223-3, Ap^r	7
pAQ5	*oxyR* wt[a] in pACYC184, Cm^r, Ap^r	34
pAQ17	*oxyR* wt in pUC12	14
pAQ25	*oxyR* wt in pKK177-3 with altered SD[b] sequence, Ap^r	35
pGSO8	*oxyR* C25S in pUC18	This study
pGSO9	*oxyR* C143S in pUC18	This study
pGSO10	*oxyR* C180S in pUC18	This study
pGSO11	*oxyR* C208S in pUC18	This study
pGSO12	*oxyR* C259S in pUC18	This study
pGSO13	*oxyR* 5CS[c] in pUC18	This study
pGSO51	*oxyR* T100I in pACYC184	This study
pGSO52	*oxyR* H114Y in pACYC184	This study
pGSO53	*oxyR* H198Y in pACYC184	This study
pGSO54	*oxyR* H198R in pACYC184	This study
pGSO55	*oxyR* R201C in pACYC184	This study
pGSO56	*oxyR* C208Y in pACYC184	This study
pGSO58	*oxyR* A233V in pACYC184	This study
pGSO59	*oxyR* A233T in pACYC184	This study
pGSO60	*oxyR* G253K in pACYC184	This study
pGSO66	*oxyR* E225K in pACYC184	20
pGSO67	*oxyR* H198R in pKK177-3	This study
pGSO68	*oxyR* C199S in pKK177-3	This study
pGSO69	*oxyR* A233V in pKK177-3	This study
pGSO70	*oxyR* E225K in pKK177-3	This study
pGSO71	*oxyR* S33N in pKK177-3	This study
pGSO72	*oxyR* C199S in pUC18	This study
pGSO73	*oxyR* C199S in pACYC184	This study

[a] wt, wild type.
[b] SD, Shine-Dalgarno.
[c] Cysteine residues 25, 143, 180, 208, and 259 changed to serine.

Primer extension assays. Cells were grown to an optical density at 600 nm of 0.4, and then half of each sample was treated with hydrogen peroxide (200 μM final concentration) for 10 min. Total RNA was isolated by using hot phenol, and 0.1 pmol of an end-labeled *oxyS* oligonucleotide (5′-GCAAAAGTTCACGT TGG) was annealed to 3 μg of total RNA as described previously (33 [short protocol]). The extension reaction was performed with Superscript reverse transcriptase from Gibco BRL (Gaithersburg, Md.) in the reaction buffer provided. The extension products were separated on an 8% sequencing gel and were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

DNase I footprinting assays. An end-labeled DNA fragment (20,000 cpm) was incubated with 1 to 2 μl of the soluble fraction of a crude cell extract or with 10 to 100 ng of pure OxyR protein in 25 μl of 0.5× TM buffer (39). Aliquots (6 pmol) of RNA polymerase (Pharmacia) were added to the binding reaction mixtures when indicated. The binding reaction mixtures were then treated with DNase I as described previously (39). The crude cell lysates were prepared by sonicating the pellet of a 5-ml overnight culture in 800 μl of 10 mM Tris buffer (pH 8) containing 20% glycerol. The soluble fraction was obtained by centrifugation.

In vitro transcription assays. The transcription assays were carried out at 37°C as described previously (35). Purified OxyR (5 pmol) was first incubated with

TABLE 2. Sensitivities of *oxyR* cysteine-to-serine mutants to oxidants

Strain	Codon exchange	Zone(s) of inhibition[a] (mm) with:		Phenotype[b]
		10% H_2O_2	4% CHP	
Vector (pUC18)		36	27	
Wild type		24 (29)	18	
C25S	TGC→TCC	24 (29)	19	Wild type
C143S	TGC→TCC	24 (29)	18	Wild type
C180S	TGC→TCC	24 (29)	18	Wild type
C199S	TGT→TCT	40	26	Noninducible
C208S	TGT→TCT	29	18	Wild type
C259S	TGC→TCC	25 (31)	17	Wild type
5CS[c]		29	17	Wild type

[a] Total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H_2O_2) or cumene hydroperoxide (CHP). The values are from a representative assay. The numbers in parentheses indicate the size of the second zone of partial growth.

[b] Phenotype of mutants with respect to their sensitivity to oxidants.

[c] In this mutant, cysteine residues 25, 143, 180, 208, and 259 were changed to serine.

pAQ17 (0.2 μg) for 10 min, and then RNA polymerase (6 pmol) was added and the reaction mixtures were incubated for another 10 min. After the addition of 1 μl of a 25 mM nucleoside triphosphate mixture, the reaction mixtures were incubated for an additional 5 min, and then the reactions were terminated by several phenol extractions. One-third of each sample was analyzed by primer extension with end-labeled *oxyS* (5′-GCAAAAGTTCACGTTGG) and *bla* (5′-AGGGCGACACGGAAATGTTGAATACTCATA) oligonucleotide primers. The primer extension assays were carried out as described above with the exception that after the extension reaction, the samples were treated with 5 μg of RNase A and subjected to a phenol extraction and ethanol precipitation.

RESULTS

Mutagenesis of cysteine residues. Previous studies have shown that only oxidized OxyR is able to activate transcription (35). To determine whether the cysteine residues in OxyR were important for sensing the oxidative stress signal, we changed each of the six cysteines to serine by site-directed mutagenesis. The mutants were cloned into pUC18 and transformed into TA4112, an *E. coli* strain carrying a chromosomal deletion of *oxyR*. These strains were then assayed for their sensitivities to hydrogen peroxide and cumene hydroperoxide by using a growth inhibition assay. As seen in Table 2, the C25S, C143S, C180S, and C259S mutant strains showed the same sensitivity as a strain carrying the wild-type *oxyR* gene. The strains showed two distinct zones of inhibition for hydrogen peroxide; the first zone corresponded to complete killing, while the second zone corresponded to partial growth. The reason for this double zone is unclear, but the C208S mutant, although resistant, showed only one zone of inhibition for hydrogen peroxide. Unlike the C25S, C143S, C180S, C208S, and C259S mutants, the C199S mutant strain was as hypersensitive to hydrogen peroxide and cumene hydroperoxide as the control strain lacking *oxyR*, suggesting that the C199S mutant was unable to induce the expression of defense genes. The C199S protein was stable and able to specifically bind DNA (see below). Therefore, we propose that the C-199 residue is the possible redox center and that the C199S mutant is locked in the reduced conformation. A mutant in which all cysteine residues except C-199 were replaced by serine showed the same resistance as the C208S single mutant.

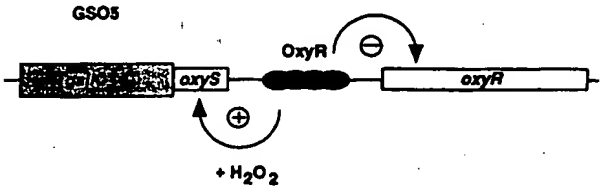

FIG. 1. *oxyS-galK* transcriptional fusion used to screen for OxyR mutants. When binding to the promoter region, OxyR represses its own expression, and upon oxidation, it activates *oxyS* transcription.

Screen for constitutively active OxyR mutants. Having identified C-199 as a critical amino acid for OxyR activity, we wanted to determine what other amino acids were required for OxyR to act as an activator. We chose to randomly mutagenize the entire *oxyR* gene and screen for OxyR mutants altered in their ability to activate transcription of the *oxyS* promoter. The *oxyS* gene encodes a small, untranslated regulatory RNA and is encoded divergently from the *oxyR* structural gene (4). The *oxyR* and *oxyS* promoters overlap, and OxyR bound to its site at these promoters acts to both repress *oxyR* expression (under oxidizing and reducing conditions) and activate *oxyS* expression (only under oxidizing conditions). We constructed a transcriptional fusion between the *oxyS* promoter and the *galK* reporter gene and integrated this fusion into the chromosome of *E. coli* SA2692 (Fig. 1). Subsequently, an *oxyR* deletion was moved into the strain by P1 transduction, giving rise to GSO5. Plasmids carrying *oxyR* were then mutagenized by hydroxylamine in vitro and introduced into the GSO5 background.

The abilities of the *oxyR* mutants to activate *oxyS* expression were monitored on MacConkey agar plates with galactose as a carbon source. For reasons that are not understood, wild-type OxyR displayed an uninducible or reduced phenotype on the MacConkey-galactose medium, even in the presence of hydrogen peroxide. The uninduced phenotype, however, suggested the possibility of screening for constitutively active mutants on this medium. We screened more than 10^6 colonies for constitutively active mutants and identified 20 candidates. The mutants were tested for their sensitivities to hydrogen peroxide and cumene hydroperoxide in a growth inhibition assay, and 18 of the mutants showed smaller zones than the wild-type strain, indicating that they were more resistant to oxidants. Only the A233V mutant consistently had the two zones of growth inhibition seen for some of the cysteine mutants. Unlike the result shown in Table 2, only a single zone was observed for the strain expressing wild-type OxyR, probably because of differences in the strain backgrounds and vectors.

Mutations map to the C-terminal part of OxyR. To determine the locations and natures of the mutations, the mutant *oxyR* genes were sequenced entirely. Eight different mutations, all causing single amino acid exchanges, were found in the pool of 18 resistant candidates (Table 3). The A233T mutation, which caused a constitutive phenotype in our random screen, affected the same alanine residue mutated (A233V) in the original *oxyR2* constitutive mutant strain (14). Similarily, two different amino acid changes at position H-198 gave rise to a constitutive phenotype.

The positions of the mutations causing the constitutive phenotype are shown on an alignment of OxyR and five other members of the LysR family in Fig. 2. The location of the noninducible C199S mutation is also indicated in Fig. 2. All of the mutations are clustered in three regions in the C-terminal two-thirds of the protein, suggesting that these regions are

TABLE 3. Sensitivities of constitutively active *oxyR* mutant strains to oxidants

Strain	Codon exchange	Zone(s) of inhibition[a] (mm) with:		Phenotype[b]
		10% H_2O_2	4% CHP	
Vector (pACYC184)		35	23	
Wild type		24	20	
C199S	TGT→TCT	42	30	Noninducible
T100I	ACA→ATA	24	12	Constitutive
H114Y	CAC→TAC	21	14	Constitutive
H198Y	CAC→TAC	21	13	Constitutive
H198R	CAC→CGC	23	14	Constitutive
R201C	CGC→TGC	23	16	Constitutive
C208Y	TGT→TAT	23	13	Constitutive
A233V[c]	GCA→GTA	21 (27)	13	Constitutive
A233T	GCA→ACA	16	12	Constitutive
G253K	GGG→AAG	19	13	Constitutive

[a] Total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H_2O_2) or cumene hydroperoxide (CHP). The values are averages from two separate assays. The number in parentheses indicates the size of the second zone of partial growth.

[b] Phenotype of mutants with respect to their color on MacConkey agar plates.

[c] This mutant corresponds to the previously isolated *oxyR2* mutant (14).

involved in sensing oxidative stress and transducing the signal to RNA polymerase. One cluster of mutations affects residues T-100 and H-114, another cluster is evident around the critical cysteine C-199, and a third is located further downstream at positions A-233 and G-253, which are at and near the original *oxyR2* mutation (A233V).

Mutants show constitutive *oxyS* transcription in vivo. The phenotype f the constitutive mutants n MacConkey agar plates suggested that, in contrast to the case for the wild-type protein, oxidation was n t required for the constitutive mutants to activate *oxyS* transcripti n. To assess whether oxidation would increase the activities of the mutants and to compare the mutants with the wild-type protein in vivo, we divided cultures expressing the different *oxyR* mutants and treated half of each culture with hydrogen peroxide. Total RNA was then isolated from both the treated and untreated cells, and the levels of the *oxyS* message were determined by primer extension. Unlike the wild-type strain, all of the untreated constitutive mutants showed *oxyS* expression (Fig. 3). Only two f the mutants (T100I and H114Y) showed a two- to threefold induction of *oxyS* expression upon treatment with hydrogen peroxide. Although all of the constitutive mutants were active under reducing conditions, the overall level of activity varied from 3 to 400% of the wild-type activity. The noninducible C199S mutant did not show any activation of *oxyS*. The hydrogen peroxide-induced expression of two additional OxyR-regulated genes, *dps* and *katG*, was also tested for a subset of the mutants (H198Y, C199S, and A233V). All of the mutants showed the same relative expression seen with the *oxyS* gene, suggesting that the individual OxyR mutants had similar effects on all target genes (data not shown).

DNA binding by OxyR constitutive mutants. We assumed that OxyR binding to its target promoters is required f r transcriptional activation. Previous studies had also shown that the oxidized and the reduced forms of OxyR have different DNA

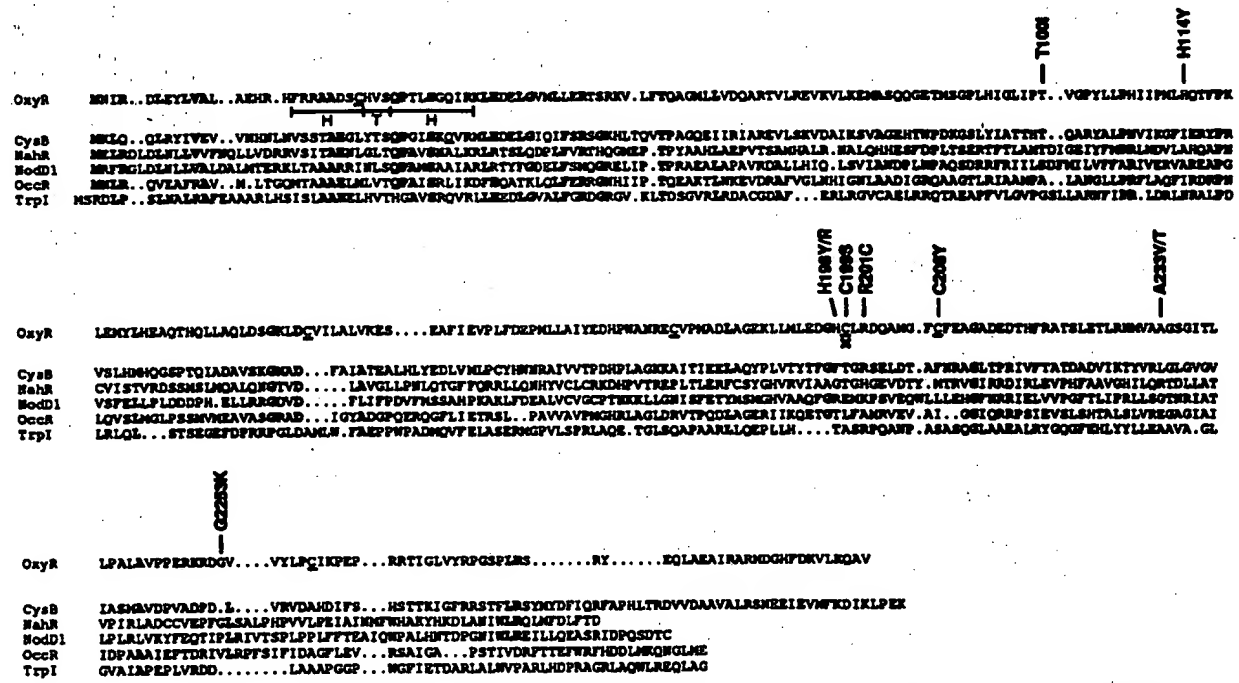

FIG. 2. Protein sequence alignment of six LysR family members and locations of the OxyR mutations causing a constitutively active phenotype. Residues that are identical in four of six sequences are in boldface, the bars mark the helix-turn-helix motif, and the six cysteine residues in OxyR are underlined. The x denotes the noninducible C199S mutant. The sequences were obtained from SWISS-PROT and GenBank (*E. coli* OxyR [OXYR_ECOLI], *E. coli* CysB [CYSB_ECOLI], *P. putida* NarR [NAHR_PSEPU], *Rhizobium meliloti* NodD1 [NOD1_RHIME], *P. aeruginosa* TrpI [TRPI_PSEAE], and *A. tumefaciens* OccR [TIPOCCR]). The alignment was done with the Genetics Computer Group program PILEUP with default parameters.

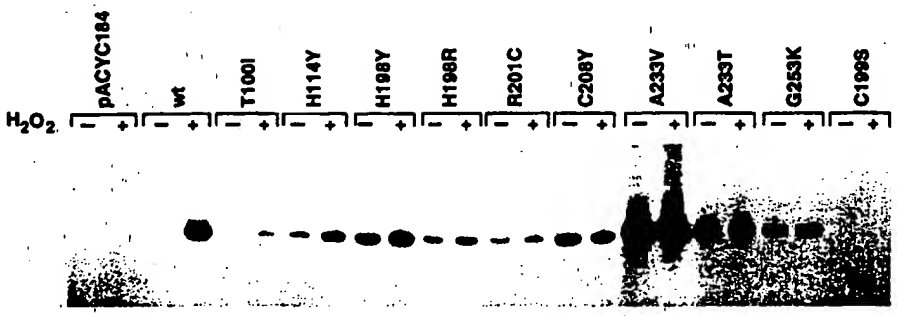

FIG. 3. Primer extension assays of *oxyS* induction in OxyR constitutive mutant strains. Total RNA was isolated from the corresponding *E. coli* strains, which were either untreated (–) or induced with 200 μM hydrogen peroxide for 10 min (+). A labeled oligonucleotide capable of hybridizing to the *oxyS* transcript was then incubated with 3 μg of total RNA and extended with reverse transcriptase. wt, wild type.

binding characteristics (35, 41). For example, at the *oxyR-oxyS* promoter, the reduced protein has a DNase I footprint that is extended compared with that of the oxidized protein. The reduced, but not the oxidized, footprint also shows a strong central DNase I-hypersensitive site. Therefore, to further characterize the mutants, we prepared cell extracts from Δ*oxyR*::*kan* strains expressing the wild-type and mutant OxyR proteins from pACYC184. We then assayed the extracts for binding to an *oxyR-oxyS* promoter fragment. If the mutations caused the proteins to be locked in the oxidized conformation, we should observe the shorter DNase I footprint characteristic of the oxidized wild-type protein. Alternatively, if the constitutive phenotype was caused only by the abnormal exposure of an activation domain, the mutants should bind in the extended conformation characteristic of reduced wild-type OxyR.

Since OxyR present in extracts prepared aerobically in the absence of high concentrations of dithiothreitol (DTT) is predominantly in the oxidized form (35), the lysate from wild-type cells gave rise to a short footprint (Fig. 4A). Among the constitutive mutants however, only G253K showed the shorter footprint. Four other constitutive mutants (H198Y, H198R, R201C, and C208Y) showed the extended DNase I footprint observed with the reduced wild-type protein. The extension was seen even when the extracts were treated with 0.2 or 2 mM hydrogen peroxide (data not shown). These results suggested that the H198Y, H198R, R201C, and C208Y proteins bind in the reduced configuration under oxidizing conditions. Reduced wild-type OxyR is not able to bind to the *katG* and *ahpC* promoter fragments, since these promoters lack determinants for reduced OxyR binding (41), and although H198R constitutively activates the *katG* and *ahpC* genes, we observed that the purified mutant protein did not give a footprint at either of these promoters (data not shown). However, the binding of RNA polymerase was found to alter the binding of H198R to the *oxyR-oxyS* fragment (see below), and an interaction with RNA polymerase may allow the H198R protein to bind to and constitutively activate the *katG* and *ahpC* promoters.

Unexpectedly, four constitutive mutants (T100I, H114Y, A233V, and A233T) behaved like E225K, a nonbinding mutant (described in reference 20), and did not show a distinct footprint even though equivalent amounts of OxyR protein were present in the samples (Fig. 4A and data not shown). Since the extracts for both the A233V and A233T mutants caused nonspecific degradation of the *oxyR-oxyS* probe, we repeated the DNase I footprint experiment with purified A233V but still did not observe a footprint (see below). These binding studies demonstrated that T100I, H114Y, A233V, and A233T have decreased apparent affinities to DNA. We did detect a weak shifted band for all four mutants in a more sensitive gel retardation assay, revealing some residual DNA binding activity which may be sufficient for activation of *oxyS* (data not shown). In addition, this weak binding must allow for autorepression by the mutant proteins, since none of the constitutively active mutants showed elevated levels of OxyR protein by immunoblot assays (data not shown). As shown below, the DNA binding affinities of some of the mutants may also be increased by the binding of RNA polymerase.

We also characterized the DNase I footprints of all the cysteine mutants created by site-directed mutagenesis (Fig. 4B). The C25S, C143S, C180S, and C259S mutants showed a short footprint, while the C199S and C208S mutants gave an extended footprint with the hypersensitive cleavage site seen with the reduced wild-type protein. The C208S mutant also showed a slightly decreased binding to the DNA compared with the wild-type protein and the other C-to-S mutants.

Transcriptional activation by mutant OxyR proteins in vitro. To study some of the different OxyR mutants in vitro, we overexpressed and purified the noninducible C199S protein and two representative constitutive mutant proteins, H198R and A233V (Fig. 5). We also overexpressed and purified two nonbinding mutants, S33N and E225K (described in the accompanying paper [20]). We then assayed the abilities of the purified wild-type and mutant proteins to activate transcription in vitro. Aliquots of the purified proteins were incubated with a supercoiled plasmid carrying the *oxyS* and *bla* (conferring ampicillin resistance) genes together with purified RNA polymerase holoenzyme in a transcription reaction. Subsequently, the in vitro-synthesized *oxyS* and *bla* transcripts were detected by primer extension assays (Fig. 6). A distinct OxyR-dependent signal was observed for *oxyS* with the wild-type protein (Fig. 6A), since aerobically purified wild-type OxyR is predominantly oxidized (35). We did not detect an *oxyS* transcript with C199S, consistent with our observations that this mutant was unable to activate transcription in vivo. The constitutive H198R mutant was nearly as active as the wild-type protein, but the constitutive A233V protein showed significantly less activity than either wild-type OxyR or H198R. The low level of activity of the A233V mutant in vitro was surprising, since this mutant is the strongest activator in vivo. Possibly the A233V protein is altered during purification or requires additional factors for stabilization (discussed in reference 20). The nonbinding mutant E225K showed very weak activity, while the second nonbinding mutant, S33N, did not show any detectable activity. The expression of the control *bla* gene was unaffected by the different OxyR mutants.

To compare the activities of the wild-type protein and the

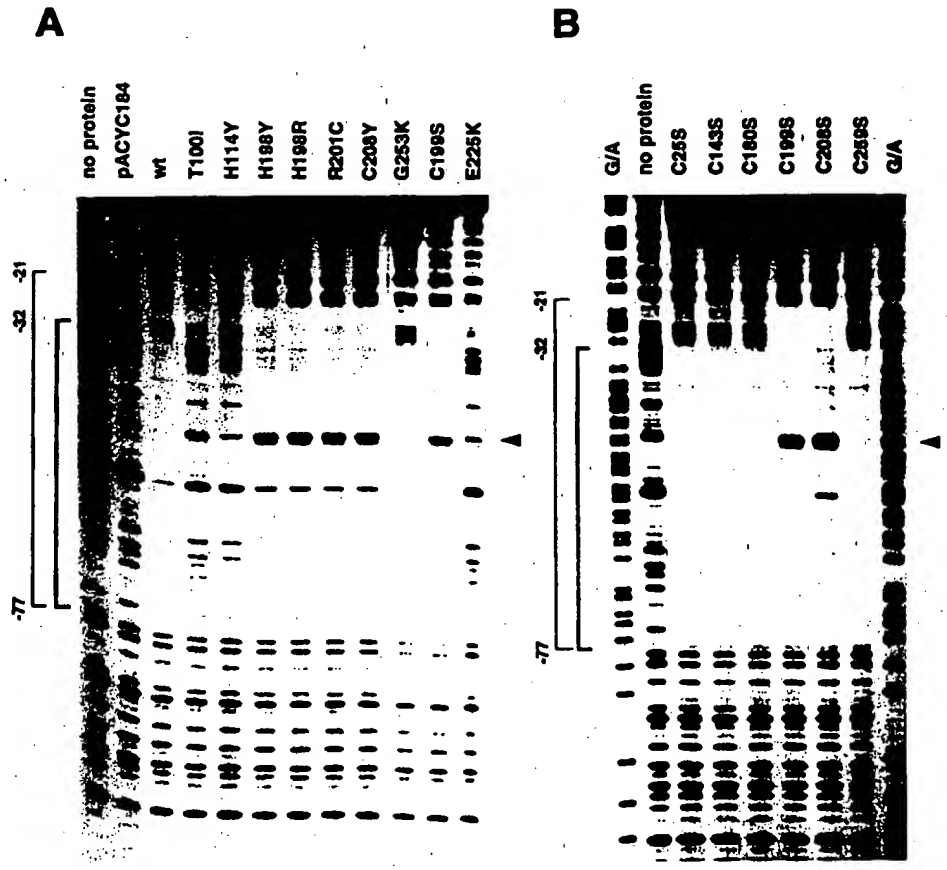

FIG. 4. DNase I footprints of the OxyR mutants binding to the top strand of the *oxyS* promoter. The 100-bp *Eco*RI-*Hin*dIII fragment of pGSO40 (41) labeled at the *Hin*dIII site was incubated with 2 μl of the soluble fraction of crude cell extracts. (A) Constitutive *oxyR* mutations carried on pACYC184; (B) cysteine mutations carried on pUC18. The heavy bracket indicates the oxidized shorter footprint of OxyR, the light bracket indicates the reduced extended footprint of OxyR, and the arrowhead indicates the hypersensitive cleavage site seen with the extended footprint. The positions of the footprints are labeled with respect to the start of the *oxyS* transcript, and the G/A sequence of the fragment is shown in panel B. wt, wild type.

constitutive mutant H198R protein under reducing conditions, the in vitro transcription assays were also carried out in presence of 200 mM DTT (Fig. 6B). For the wild-type protein, the activity under reducing conditions dropped to 12% of the activity in the absence of DTT, while for the H198R mutant, the activity was only reduced to 48% of the activity in the absence of DTT, showing that the H198R protein is less sensitive to reducing conditions. These observations agree with our findings that H198R is active under normal, reducing conditions in vivo.

Cooperative binding between mutant OxyR proteins and RNA polymerase. Tao and colleagues have shown that OxyR has a cooperative effect on RNA polymerase binding to the *katG* promoter site (36). To test whether the mutant proteins affected polymerase binding, we analyzed the DNase I footprint of RNA polymerase binding to the *oxyR-oxyS* promoter fragment in the presence of the purified OxyR mutants (Fig. 7A). RNA polymerase (0.3 to 10 pmol) alone did not show a footprint at the *oxyS* promoter (Fig. 7A, lane 2). However, when the polymerase was incubated with oxidized wild-type OxyR, a clear protection due to polymerase was observed at the *oxyS* promoter (Fig. 7A, lane 4). The uninducible mutant, C199S, showed an extended footprint alone (Fig. 7A, lane 5)

FIG. 5. Overexpression and purification of OxyR mutant proteins. Aliquots of uninduced and induced cultures, the soluble fraction of induced cells (described in Materials and Methods), and purified protein fractions (80 to 100 μg) were electrophoresed on an SDS–12% PAGE gel and stained with Coomassie blue. The A233V and E225K proteins showed reduced affinities to the heparin and eluted at lower salt concentrations than the wild-type protein, which likely accounts for the contaminating protein band in these samples. The mobilities of the standard proteins are indicated to the left of the gel. wt, wild type.

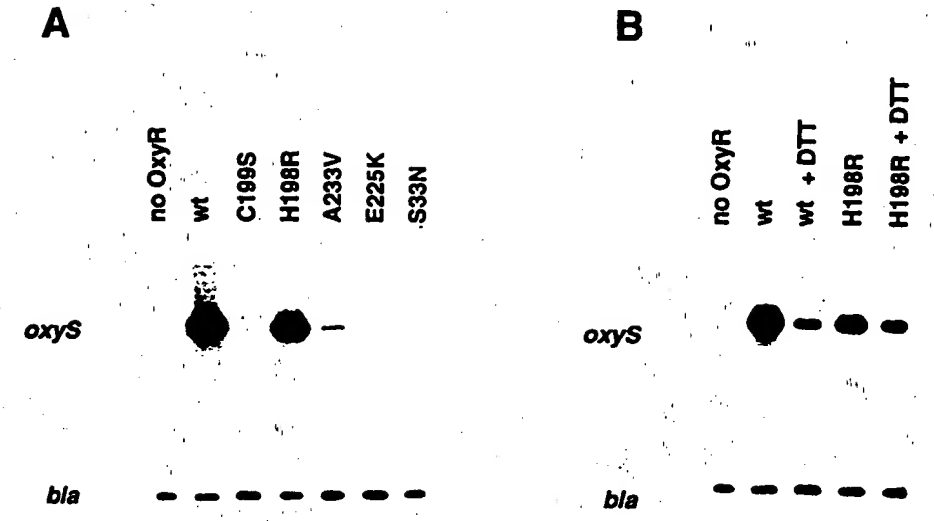

FIG. 6. In vitro transcription assays of purified wild-type (wt) and OxyR mutant proteins. The transcription reactions were carried out as described in Materials and Methods. The transcripts were then detected with *oxyS*- and *bla*-specific oligonucleotides in a primer extension assay. (A) Assays with the purified wild-type and C199S, H198R, A233V, E225K, and S33N mutant proteins under oxidizing conditions (no DTT); (B) assays with the wild-type and constitutive H198R mutant proteins in the absence or presence of 200 mM DTT.

and did not induce RNA polymerase binding (lane 6), consistent with the lack of transcription activation seen with this mutant. In contrast, the constitutive H198R mutant, which also showed an extended footprint alone (Fig. 7A, lane 7), had a cooperative effect on polymerase binding (lane 8). Interestingly, the strong DNase I-hypersensitive site detected for H198R alone was not seen in the presence of polymerase. The A233V constitutive mutant, which did not bind to the DNA alone (Fig. 7A, lane 9), nevertheless stimulated RNA polymerase binding (lane 10), although the binding was weaker than that observed with H198R. The nonrepressing mutants E225K and S33N (described in the accompanying paper [20]) also did not bind to DNA alone (Fig. 7A, lanes 11 and 13), but E225K could still induce some cooperative binding of RNA polymerase (lane 12). These footprints showed that the constitutive mutants (H198R and A233V), but not the noninducible mutant (C199S), had a cooperative effect on RNA polymerase binding to the *oxyS* promoter. The studies also showed that an interaction with RNA polymerase increased the DNA binding affinity of the A233V and E225K mutant proteins.

We examined RNA polymerase binding with wild-type OxyR and H198R in the presence of 200 mM DTT (Fig. 7B). The wild-type protein did not increase the binding of polymerase under the reducing conditions (Fig. 7B, lane 7), while the constitutively active H198R mutant still had a cooperative effect on polymerase in the presence of the 200 mM DTT (lane 11). These observations support the conclusion that H198R is constitutively active and that the ability to induce RNA polymerase-promoter binding correlates with the ability to activate transcription.

DISCUSSION

In this study, we used site-directed and random mutageneses to isolate one noninducible and eight constitutively active OxyR mutants. Together with the nonbinding mutants described in the accompanying paper (20), these mutants have allowed us to define functional domains in the OxyR protein.

C-199 as a possible redox center. Using site-directed mutagenesis, we found that only one (C-199) of six cysteine residues was critical for the induction of defense genes by OxyR. The C199S mutant was unable to activate the antioxidant genes and gave rise to an extended footprint characteristic of reduced OxyR. Since oxidation is necessary for OxyR to activate transcription in vitro, we suggest that C-199 is the redox center of the protein and that the C199S protein is locked in the reduced conformation. Cysteines are known to be redox-reactive amino acids and can form inter- or intramolecular disulfide bridges. However, an intramolecular disulfide bridge is unlikely to be the redox-active center of OxyR, since only C-199 proved to be critical, and the mutant in which all other cysteines were mutated to serine was still active in vivo. We did not observe any differences between the wild-type and mutant C199S proteins on nonreducing gels which permit the resolution of disulfide-linked oligomers (data not shown), suggesting that intermolecular disulfide bridges do not constitute the redox-active center. Redox-active metals are also coordinated by cysteine residues. The SoxR protein, a transcriptional activator in the bacterial response to superoxide, carries a nonheme iron which is likely to be the redox-reactive center of the SoxR protein (17). However, we do not think that a metal coordinated through C-199 is the redox-active center of OxyR, since the addition of chelators did not affect transcriptional activation of OxyR in vitro (35). Cysteines have been shown to be reversibly oxidized to sulfenic (SO^-) or sulfinic (SO_2^-) acid and irreversibly oxidized to sulfonic (SO_3^-) acid. Both a streptococcal NADH peroxidase and an oxidation product of the protease papain have been reported to have a cysteine-sulfenic acid (22, 27). Since OxyR is reversibly oxidized and only a single cysteine is critical for OxyR activity, we suggest that C-199 is oxidized to a sulfenic acid. The mutants described

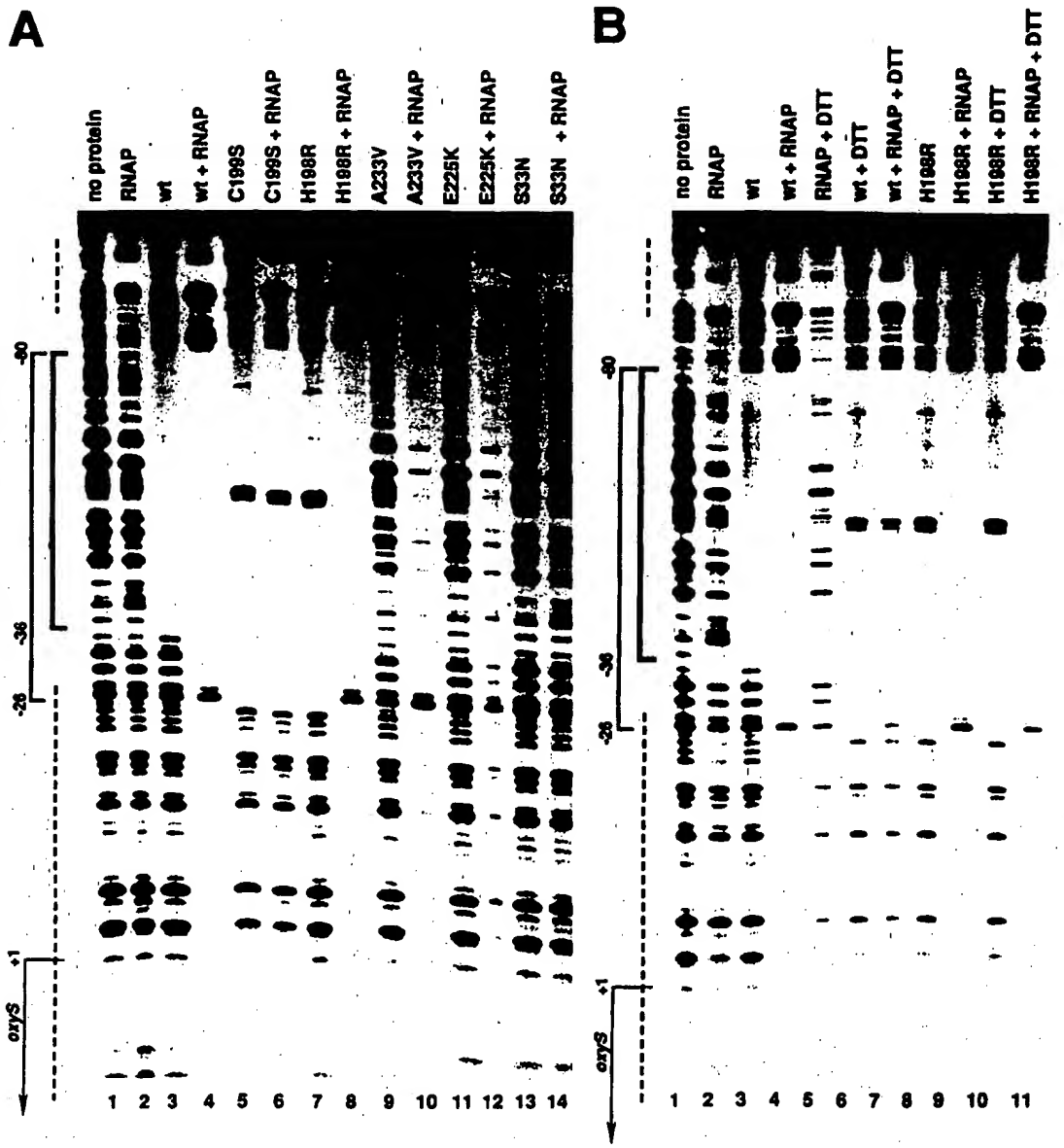

FIG. 7. DNase I footprints of RNA polymerase (RNAP) together with wild-type (wt) and mutant OxyR proteins on the bottom strand of the *oxyS* promoter fragment. The 200-bp *Eco*RI-*Hin*dIII fragment of pGSO43 (41) labeled at the *Eco*RI site was incubated with purified RNA polymerase and the purified wild-type and OxyR mutant proteins as indicated. (A) Footprinting assays carried out in the absence of DTT; (B) RNA polymerase binding assayed in the absence or presence of 200 mM DTT. The heavy brackets denote the oxidized footprint of OxyR, the light brackets denote the reduced extended footprint of OxyR, and the dotted lines indicate the sequences protected by RNA polymerase binding. The positions of the footprints are labeled with respect to the start of the *oxyS* transcript. The upper protection by RNA polymerase does not correspond to either the *oxyS* or *oxyR* promoter and is assumed to be due to nonspecific binding to the end of the fragment.

here will be useful for future biochemical studies of the modifications of the cysteine residues in OxyR.

Constitutively active mutants define inducer-responsive and activation domains. Using a random mutagenesis approach, we isolated eight mutants which constitutively activate transcription of their target genes during normal growth and show increased resistance to oxidants. The corresponding mutations can be grouped and map to domains that may be functionally conserved among the LysR family members. Two of the amino acid substitutions (T100I and H114Y) map to a region (residues 98 to 150) that is somewhat conserved among the LysR proteins and that has been defined as a coinducer recognition/response domain by mutations in other LysR family members (29). Four of the mutations (H198Y, H198R, R201C, and C208Y) map within or near a second coinducer recognition/response domain (residues 196 to 206) of the LysR family (29). These four mutations are located close to the proposed redox-active C-199 residue, consistent with the conclusion that this region is the hydrogen peroxide-responsive domain. Finally, three of the mutations (A233V, A233T, and G253K) are lo-

cated near a carboxy-terminal region (residues 227 to 253) that shows some homology between the LysR proteins. Given the partial conservation of residues 227 to 253 and residues 98 to 150, these regions might be involved in touching RNA polymerase. Experiments described in the accompanying paper also indicate that the C-terminal domain is involved in oligomerization of OxyR (20).

It is not clear whether the critical C-199 residue is still modified by oxidation in the constitutive mutants. However, since the T100I and H114Y mutants still showed a two- to threefold increase of activation upon oxidation, these mutants should still be redox reactive. Three of the mutations causing a constitutively active phenotype affected histidine residues (H114Y, H198Y, and H198R). The crystal structures of the streptococcal NADH peroxidase and the oxidized papain (the two proteins found to have a sulfenic acid) showed that histidine residues form hydrogen bonds with the redox-active cysteine (19, 32). If a sulfenic acid proves to be the redox center of OxyR, the H-114 and H-198 residues may act to form hydrogen bonds with the sulfenic acid. We suggest that the amino acids of the constitutive mutants may more effectively stabilize the negatively charged residue and allow the oxidation of C-199 during normal growth. Structural studies of OxyR should help to elucidate whether the regulator carries a sulfenic acid stabilized by histidine residues and give additional insights into the domains of OxyR and other LysR family proteins.

Cooperative binding with RNA polymerase. The overall activities of the constitutive mutants varied from 3 to 400% of the wild-type activity in vivo. It was not possible to correlate the variations in activity with differences in DNA binding. In fact, we were surprised to find that although one constitutive mutant gave rise to a short oxidized footprint, several constitutive mutants showed reduced extended footprints, and some of the mutants showed no footprint at all. The different footprint phenotypes were specific to different regions, since both T100I and H114Y had decreased binding and all of the mutants with substitutions near C-199, including the C208S mutant generated by site-directed mutagenesis, gave extended footprints. The ability to activate transcription, however, did correlate with the abilities of the purified proteins to bind cooperatively with RNA polymerase. The uninducible C199S mutant did not induce transcription and was unable to stimulate RNA polymerase binding. In contrast, the constitutive H198R mutant, which showed the same extended reduced footprint seen for C199S in the absence of RNA polymerase, induced polymerase binding even in the presence of 200 mM DTT. Another constitutive mutant, A233V, showed significantly reduced binding to DNA alone but also induced polymerase binding.

RNA polymerase binding to the promoter can be a key step in the activation by transcription factors. At some bacterial promoters, RNA polymerase is unable to bind in the absence of a transcriptional regulator, preventing the titration to these promoters when the corresponding genes need not be activated. For example, RNA polymerase is unable to bind to the promoters of the *pho* (phosphate) genes in the absence of the PhoB activator (23). We propose that the oxidation of the wild-type OxyR protein exposes a domain which allows OxyR to recruit RNA polymerase and that the constitutive phenotype of our mutants may be due to exposure of this domain under both oxidizing and reducing conditions.

Our results also suggest that while OxyR induces RNA polymerase binding, RNA polymerase has an effect on OxyR. Both A233V and E225K did not shown DNase I footprints when incubated with the *oxyS* promoter alone but did bind in the presence of RNA polymerase. We propose that the contact with polymerase may also cause the H198R mutant to adopt the oxidized configuration, since the DNase I-hypersensitive site in the center of the reduced H198R footprint disappears upon the interaction with RNA polymerase. An important direction for further studies will be to elucidate this reciprocal interaction between OxyR and RNA polymerase.

ACKNOWLEDGMENTS

We thank S. Adhya for strains and plasmids, and we thank S. Altuvia, S. Garges, R. Klausner, and C. Wu for helpful discussions and comments on the manuscript.

I.K. was the recipient of fellowships from the Schweizerischen Nationalfonds and the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Adhya, S. Unpublished data.
2. Adhya, S., and S. Garges. 1990. Positive control. J. Biol. Chem. 265:10797–10800.
3. Altuvia, S., M. Almirón, G. Huisman, R. Kolter, and G. Storz. 1994. The *dps* promoter is activated by OxyR during growth and by IHF and σ^s during stationary phase. Mol. Microbiol. 13:265–272.
4. Altuvia, S., and G. Storz. Unpublished data.
5. Bolivar, F., and K. Backman. 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. 68:245–267.
6. Bölker, M., and R. Kahmann. 1989. The *Escherichia coli* regulatory protein OxyR discriminates between methylated and unmethylated states of the phage Mu *mom* promoter. EMBO J. 8:2403–2410.
7. Brosius, J., and A. Holy. 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA 81:6929–6933.
8. Bullock, W. O., J. M. Fernandez, and J. M. Short. 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques 5:376.
9. Burn, J. E., W. D. Hamilton, J. C. Wootton, and A. W. B. Johnston. 1989. Single and multiple mutations affecting properties of the regulatory gene *nodD* of *Rhizobium*. Mol. Microbiol. 3:1567–1577.
10. Calos, M. P. 1978. DNA sequence for a low-level promoter of the *lac* repressor gene and an "up" promoter mutation. Nature (London) 274:762–765.
11. Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141–1156.
12. Chang, M., and I. P. Crawford. 1990. The roles of indoleglycerol phosphate and the TrpI protein in the expression of *trpBA* from *Pseudomonas aeruginosa*. Nucleic Acids Res. 18:979–988.
13. Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell 41:753–762.
14. Christman, M. F., G. Storz, and B. N. Ames. 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA 86:3484–3488.
15. Davis, R. W., D. Botstein, and J. R. Roth. 1980. Advanced bacterial genetics: a manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. Fisher, R. F., and S. R. Long. 1992. *Rhizobium*-plant signal exchange. Nature (London) 357:655–660.
17. Hidalgo, E., and B. Demple. 1994. An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. EMBO J. 13:138–146.
18. Huang, J., and M. A. Schell. 1991. *In vivo* interactions of the NahR transcriptional activator with its target sequences: inducer-mediated changes resulting in transcription activation. J. Biol. Chem. 266:10830–10838.
19. Kamphuis, I. G., K. H. Kalk, M. B. A. Swarte, and J. Drenth. 1984. Structure of papain refined at 1.65 Å resolution. J. Mol. Biol. 179:233–256.
20. Kullik, I., J. Stevens, M. B. Toledano, and G. Storz. 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. J. Bacteriol. 177:1285–1291.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
22. Lin, W. S., D. A. Armstrong, and G. M. Gaucher. 1975. Formation and repair of papain sulfenic acid. Can. J. Biochem. 53:298–307.
23. Makino, K., M. Amemura, S.-K. Kim, A. Nakata, and H. Shinagawa. 1993. Role of the σ^{70} subunit of RNA polymerase in transcriptional activation by activator protein PhoB in *Escherichia coli*. Genes Dev. 7:149–160.
24. McIver, J., M. A. Djordjevic, J. J. Weinman, G. L. Bender, and B. G. Rolfe. 1989. Extension of host range of *Rhizobium leguminosarum* bv. *trifolii* caused by point mutations in *nodD* that result in alterations in regulatory function and recognition of inducer molecules. Mol. Plant-Microbe Interact. 2:97–106.

25. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. Ostrowski, J., and N. M. Kredich. 1989. Molecular characterization of the *cysJIH* promoters of *Salmonella typhimurium* and *Escherichia coli*: regulation by *cysB* protein and *N*-acetyl-L-serine. J. Bacteriol. 171:130–140.
27. Poole, L. B., and A. Claiborne. 1989. The non-flavin redox center of the streptococcal NADH peroxidase. J. Biol. Chem. 264:12330–12338.
28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. Schell, M. A. 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. 47:597–626.
30. Schell, M. A., P. H. Brown, and S. Raju. 1990. Use of saturation mutagenesis to localize probable functional domains in the NahR protein, a LysR-type transcription activator. J. Biol. Chem. 265:3844–3850.
31. Spaink, H. P., C. A. Wijffelman, R. J. H. Okker, and B. E. J. Lugtenberg. 1989. Localization of functional regions of the *Rhizobium nodD* products using hybrid *nodD* genes. Plant Mol. Biol. 12:59–73.
32. Stehle, T., S. A. Ahmed, A. Claiborne, and G. E. Schulz. 1991. Structure of NADH peroxidase from *Streptococcus faecalis* 10C1 refined at 2.16 Å resolution. J. Mol. Biol. 221:1325–1344.
33. Storz, G., and S. Altuvia. 1994. OxyR regulon. Methods Enzymol. 234:217–223.
34. Storz, G., M. F. Christman, H. Sies, and B. N. Ames. 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA 84:8917–8921.
35. Storz, G., L. A. Tartaglia, and B. N. Ames. 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science 248: 189–194.
36. Tao, K., N. Fujita, and A. Ishihama. 1993. Involvement of the RNA polymerase α subunit C-terminal region in co-operative interaction and transcriptional activation with OxyR protein. Mol. Microbiol. 7:859–864.
37. Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa. 1989. Molecular cloning and nucleotide sequencing of *oxyR*, the positive regulatory gene of a regulon for an adaptive response to oxidative stress in *Escherichia coli*: homologies between OxyR protein and a family of bacterial activator proteins. Mol. Gen. Genet. 218:371–376.
38. Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa. 1991. Purification and characterization of the *Escherichia coli* OxyR protein, the positive regulator for a hydrogen peroxide-inducible regulon. J. Biochem. 109:262–266.
39. Tartaglia, L. A., G. Storz, and B. N. Ames. 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. J. Mol. Biol. 210:709–719.
40. Thomas, M. S., and R. E. Glass. 1991. *Escherichia coli rpoA* mutation which impairs transcription of positively regulated systems. Mol. Microbiol. 5:2719–2725.
41. Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz. 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA binding site: a mechanism for differential promoter selection. Cell 78:897–909.
42. Wang, L., J. D. Helmann, and S. C. Winans. 1992. The *A. tumefaciens* transcriptional activator OccR causes a bend at a target promoter, which is partially relaxed by a plant tumor metabolite. Cell 69:659–667.
43. Warne, S. R., J. M. Varley, G. J. Boulnois, and M. G. Norton. 1990. Identification and characterization of a gene that controls colony morphology and auto-aggregation in *Escherichia coli* K12. J. Gen. Microbiol. 136:455–462.

JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1285–1291
0021-9193/95/$04.00+0

Vol. 177, No. 5

Mutational Analysis of the Redox-Sensitive Transcriptional Regulator OxyR: Regions Important for DNA Binding and Multimerization

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Received 26 July 1994/Accepted 15 December 1994

OxyR is a LysR-type transcriptional regulator which negatively regulates its own expression and positively regulates the expression of proteins important for the defense against hydrogen peroxide in *Escherichia coli* and *Salmonella typhimurium*. Using random mutagenesis, we isolated six nonrepressing OxyR mutants that were impaired in DNA binding. Five of the mutations causing the DNA binding defect mapped near the N-terminal helix-turn-helix motif conserved among the LysR family members, confirming that this region is a DNA binding domain in OxyR. The sixth nonrepressing mutant (with E-225 changed to K [E225K]) was found to be predominantly dimeric, in contrast to the tetrameric wild-type protein, suggesting that a C-terminal region defined by the E225K mutation is involved in multimerization.

The *Escherichia coli* OxyR protein is a redox-sensitive transcriptional regulator which activates the expression of antioxidant defense genes under oxidizing conditions. During normal growth and upon oxidative stress, OxyR also acts as a repressor and negatively autoregulates its own expression and the expression of the Mu phage *mom* gene (8, 16, 34). OxyR specifically binds upstream of the promoters it regulates, but the seven natural binding sites which have been characterized only show limited homology (35). Recent studies of 54 synthetic binding sites, however, allowed the definition of an oxidized-OxyR binding motif composed of four ATAGxt elements (37). OxyR-DNase I footprints are long and cover 45 bp, and hydroxyl radical footprinting and interference assays showed that the oxidized OxyR protein binds to the four ATAGxt elements by contacting the DNA in four adjacent major grooves (37). These footprinting assays also showed that OxyR binding is different under oxidizing and reducing conditions (32, 37).

OxyR is a member of the family of LysR-type transcriptional regulators (8, 16, 33, 39). LysR family members are DNA-binding proteins which positively regulate expression of their target genes and often also negatively regulate their own expression (reviewed in reference 28). Sequence comparisons among LysR family members have shown that the region encompassing the 66 N-terminal amino acids exhibits the greatest sequence identity and includes a helix-turn-helix (HTH) motif likely to be a DNA binding domain (28). Mutations which map to the HTH region lead to a loss of DNA binding by *Pseudomonas putida* NahR (29), *Rhizobium leguminosarum* NodD (11), and other LysR-type proteins. Parts of the C-terminal domains of LysR-type proteins also seem to contribute to DNA binding, since several mutations in this region of *P. putida* NahR (29) and *Citrobacter freundii* AmpR (6) affect DNA binding.

Like OxyR, other LysR family members protect unusually long regions from DNase I digestion. The long binding sites suggest that the LysR proteins may be multimeric, and *E. coli* MetR (25), *Rhizobium meliloti* NodD3 (17), and *Klebsiella aerogenes* Nac (19) have been reported to be dimers, while NahR (29), *Pseudomonas aeruginosa* TrpI (14), and *Salmonella typhimurium* CysB (26) have been found to be tetramers. The regions involved in multimerization are not yet well defined; however, some of the C-terminal mutations in NahR and also in AmpR are not negative *trans* dominant, suggesting that they might affect a multimerization domain (6, 29).

In this study, we used random mutagenesis to define regions of the OxyR protein involved in DNA binding. We were able to show that the proposed HTH motif in OxyR is a DNA binding domain, since several mutations in this region led to impaired DNA binding of the mutant proteins. Moreover, we found that the oxidized and reduced forms of OxyR are predominantly tetrameric, while one binding mutant and one constitutively active mutant, described in the accompanying paper (21), were dimeric, suggesting that the C-terminal region affected by these mutations is involved in tetramerization.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are described in Table 1. The strain GSO7 was constructed as follows. The 0.2-kb *Hin*dII-*Ssp*I fragment of pAQ17 carrying the *oxyS* and *oxyR* promoter region (16) was cloned into the unique *Sma*I site of pTS7 (1) to create the *galK* fusion. The plasmid was then recombined onto λ_{VZ05S} (c1857 *galK nin5*) and integrated into the *att*λ site of SA2692. A Δ*oxyR*::*kan* deletion-insertion mutation was subsequently moved into the strain by P1 transduction (3). The pACYC184 derivative pAQ5 used for the mutagenesis contains *oxyR* on a *Bam*HI-*Eco*RV fragment (31). A *Bam*HI-*Hin*dIII fragment of these clones was moved into M13mp18 for sequencing. All sequencing and subcloning were carried out by standard procedures.

Media and growth conditions. Strains were grown in LB medium (27), and ampicillin (100 μg/ml [final concentration]), kanamycin (25 μg/ml), chloramphenicol (25 μg/ml), or tetracycline (15 μg/ml) was added when appropriate. The resistance of strains to hydrogen peroxide and cumene hydroperoxide was assayed by zones of inhibition, which were determined as described previously (15) except that the strains were grown in and plated on LB medium containing the appropriate antibiotics.

Mutagenesis. Approximately 10 μg of purified pAQ5 plasmid DNA was randomly mutagenized with hydroxylamine as described in the accompanying paper (21). The mutagenized DNA mixture (5 to 10 μl) was then used to transform *E. coli* XL1-blue cells directly. The transformants were rinsed off the plates, and the plasmid DNA was isolated and used to transform the GSO7 recipient strain.

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
XL1-blue	F' [*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)]	10
D1210	HB101/F' [*lacI*^{q}]	7
TA4484	*oxyR*Δ*3*, pMC7	36
SA2692	HB101 *recA*$^+$ Δ*lac* Δ*gal-165*	1
GSO7	SA2692 Δ*oxyR*::*kan* (λ_{RS055} *oxyR-galK*)	This study
GSO27	SA2692 (λ_{RS055} *oxyR-galK*)	This study
Plasmids		
pTS7	pBR322 *int* P'P; promoterless *lacZ* and *galETK*, Ap^r	1
pMC7	*lacI*^{q} Tc^r	12
pACYC184	Cm^r Tc^r	13
pKK177-3	P_{tac} promoter, derivative of pKK223-3, Ap^r	9
pAQ5	*oxyR* wt[a] in pACYC184, Cm^r	31
pAQ25	*oxyR* wt in pKK177-3 with altered SD[b] sequence, Ap^r	32
pGSO61	*oxyR* R4C in pACYC184	This study
pGSO62	*oxyR* T31M in pACYC184	This study
pGSO63	*oxyR* L32F in pACYC184	This study
pGSO64	*oxyR* S33N in pACYC184	This study
pGSO65	*oxyR* R50W in pACYC184	This study
pGSO66	*oxyR* E225K in pACYC184	This study
pGSO68	*oxyR* C199S in pKK177-3	21
pGSO69	*oxyR* A233V in pKK177-3	21
pGSO70	*oxyR* E225K in pKK177-3	21

[a] wt, wild type.
[b] SD, Shine-Dalgarno.

Finally, the GSO7 transformants were screened for the desired phenotype on MacConkey agar plates.

Primer extension assays. Cells were grown to an optical density at 600 nm of 0.4, and then half of each sample was treated with hydrogen peroxide (200 μM final concentration) for 10 min. Total RNA was isolated by using hot phenol, and 0.1 pmol of an end-labeled *oxyS* oligonucleotide (5'-GCAAAAGTTCACGT TGG) was annealed to 3 μg of total RNA as described previously (30 [short protocol]). The extension reaction was performed with Superscript reverse transcriptase from Gibco BRL (Gaithersburg, Md.) in the reaction buffer provided. The extension products were separated on an 8% sequencing gel and were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Immunoblot assays. Proteins were separated on a sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis gel (22) and transferred to a nitrocellulose filter by electroblotting. The filter was then probed with a 1:10,000 dilution of antibodies to an OxyR–β-galactosidase fusion protein (32). Bound antibody was visualized with rabbit antiserum by using the enhanced chemiluminescence Western blotting (immunoblotting) system from Amersham, Arlington Heights, Ill.

DNA binding assays. Binding reactions were carried out as described previously with 5,000 cpm of an end-labeled DNA fragment and either 1 to 10 ng of pure protein (21) or 1 to 2 μl of the soluble fraction of a crude cell lysate (36). For the mobility shift assays, the binding reaction mixtures were loaded on a nondenaturing, low-ionic-strength, 8% polyacrylamide gel (5). To prepare the crude cell extract, the pellet from a 5-ml overnight culture was sonicated in 800 μl of 10 mM Tris buffer (pH 8) containing 20% glycerol. The insoluble fraction was then removed by centrifugation.

Galactokinase assays. The samples for the galactokinase assays were obtained as described previously (2). The assays were carried out as described by Wilson and Hogness (40). D-[1-^{14}C]galactose (55 mCi/mmol; 200 Ci/ml) was obtained from Amersham, and the ion-exchange filter paper (DE81) was obtained from Whatman (Maidstone, England).

Gel chromatography. About 1 to 2 mg of pure protein (21) mixed with standard proteins (Bio-Rad, Richmond, Calif.) in a 200-μl volume was applied to a Superose 12 gel filtration column (Pharmacia, Piscataway, N.J.) equilibrated with buffer Z (21) containing 0.3 M KCl. The proteins were eluted with the same buffer Z, and aliquots of the column fractions were analyzed by immunoblots.

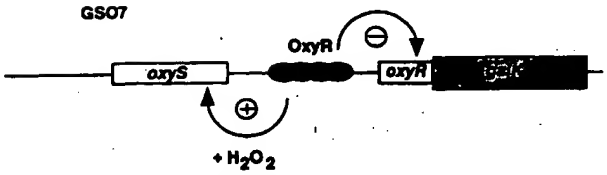

FIG. 1. *oxyR-galK* transcriptional fusion used to screen for OxyR nonrepressing mutants. OxyR bound to the *oxyR-oxyS* promoter regio. represses its own expression under both oxidizing and reducing conditions and activates *oxyS* transcription upon oxidation.

RESULTS

Screen for nonrepressing OxyR mutants. To characterize OxyR domains required for DNA binding, we chose to randomly mutagenize the entire *oxyR* gene and screen for mutants defective in DNA binding. To easily identify mutants, we constructed a transcriptional fusion between the *oxyR* promoter and the *galK* reporter gene. When OxyR binds to this site at the overlapping *oxyR* and *oxyS* promoters (Fig. 1), OxyR represses the expression of the *oxyR* gene independent of the oxidative state of the protein. Under oxidizing conditions, OxyR also acts to induce expression of the divergently transcribed *oxyS* gene, which encodes a small, untranslated regulatory RNA (4). Since binding of the OxyR protein to the DNA is assumed to be required for repression, mutant OxyR proteins which are not able to repress *oxyR* expression are likely to be DNA-binding mutants. The *oxyR-galK* fusion was integrated into the chromosome of *E. coli* SA2692, and subsequently a Δ*oxyR*::*kan* deletion was moved into the strain by P1 transduction to generate GSO7. Plasmids carrying the *oxyR* gene were mutagenized by hydroxylamine in vitro and introduced into this background. The abilities of the different mutants to repress *oxyR* expression were monitored on MacConkey agar plates containing galactose as a carbon source. In GSO7, wild-type OxyR repressed the expression of the *oxyR-galK* fusion, resulting in white colonies, while mutants that were unable to repress this fusion were detected as red colonies.

More than 10^6 colonies were screened for nonrepressing mutants with the *oxyR-galK* fusion strain, and 32 candidates were isolated. Since a truncated or unstable form of OxyR would also lead to red colonies in GSO7, we checked wh ther crude extracts of strains harboring the corresponding *oxyR* mutations expressed a full-length OxyR protein. Ten of the 32 candidates did not show any detectable OxyR protein in an immunoblot assay, and one of the candidates expressed a truncated protein. The other 21 candidates showed a full-length protein, and the expression of OxyR was elevated approximately 50-fold compared with that in the wild-type strain (see below), consistent with the observation that these mutants are unable to act as repressors of the *oxyR* promoter.

The mutants were then tested for their sensitivities to hydrogen peroxide and cumene hydroperoxide in a growth inhibition assay. All showed a larger killing zone than the wild-type strain, indicating that they were more sensitive to the oxidants than the wild-type strain (Table 2). The increased sensitivity t oxidants, and also to peroxides presumed to be in the medium, most likely accounts for the impaired ability of the mutant strains to form single colonies. As observed for the Δ*oxyR*::*kan* strain, the single colonies grew only near heavy streaks of cells.

Five nonrepressing mutations map near the HTH motif. T determine the locations and natures of the mutations, the mutant *oxyR* genes were sequenced entirely. Six different mutations, all causing single amino acid changes, were found

TABLE 2. Sensitivities of nonrepressing *oxyR* mutant strains to oxidants

Strain[a]	Codon exchange	Zone of inhibition[b] (mm) with:	
		10% H_2O_2	4% CHP
Vector (pACYC184)		35	23
Wild type		24	20
R4C	CGT→TGT	41	34
T31M	ACG→ATG	41	26
L32F	CTT→TTT	33	25
S33N	AGC→AAC	43	33
R50W	CGG→TGG	32	21
E225K	GAA→AAA	26	22

[a] All mutants had a nonrepressing phenotype with respect to their color on MacConkey agar plates.

[b] Total diameter of the zone of inhibition caused by the addition of hydrogen peroxide (H_2O_2) and cumene hydroperoxide (CHP). The values are averages from two separate assays.

among the 21 nonrepressing candidates (Table 2). With one exception, all of the nonrepressing mutations were located in or within 15 amino acids of the HTH motif of the OxyR protein presumed to be involved in DNA binding (Fig. 2). The region around the HTH motif displays the highest level of sequence similarity among LysR-type proteins, and amino acid S-33 is one of the most conserved amino acids. We found that the mutant with a change of S-33 to N (S33N) had a very severe mutant phenotype and was even more sensitive to hydrogen peroxide and cumene hydroperoxide than the Δ*oxyR*::*kan* strain. The mutations R4C and R50W also affect strongly conserved amino acids. One mutation, E225K, is not located near the HTH motif, and the failure of this mutant to repress *oxyR* expression is thought to be due to a defect in multimerization of the protein (see below).

One nonrepressing mutant protein was sequenced even though immun blot analysis revealed the protein to be slightly smaller than the wild-type OxyR protein. This mutant carried an amber mutation at position 283 and was not able to complement an *oxyR* deletion strain (data not shown). Since a C-terminal deletion of only 22 amino acids resulted in a nonrepressing phenotype, this region may be important for DNA binding and may contact the DNA, be required for multimerization, or be crucial for the appropriate folding of the protein. A previously described strain expressing OxyR with a deletion of only 11 C-terminal amino acids showed a wild-type phenotype in the peroxide sensitivity assay, suggesting that this truncated protein is still able to bind DNA (data not shown) (16).

Nonrepressing mutants show decreased expression of defense genes. The ability of OxyR to bind DNA was assumed to be required for both repression and activation of transcription. Since all of the nonrepressing mutants exhibited increased sensitivity to oxidants, we examined whether the mutant proteins were impaired in activating transcription of defense genes in vivo and whether the activity of the mutants would still be affected by oxidation. Total RNA was isolated from untreated and hydrogen peroxide-treated cultures of strains expressing the different nonrepressing mutants. The levels of the OxyR-activated *oxyS* message were then determined by primer extension assays (Fig. 3). As expected, some of the mutants (T31M, S33N, and R50W) showed no activation of *oxyS*. Three mutants (R4C, L32F, and E225K), however, could still activate transcription under oxidizing conditions, although the abilities

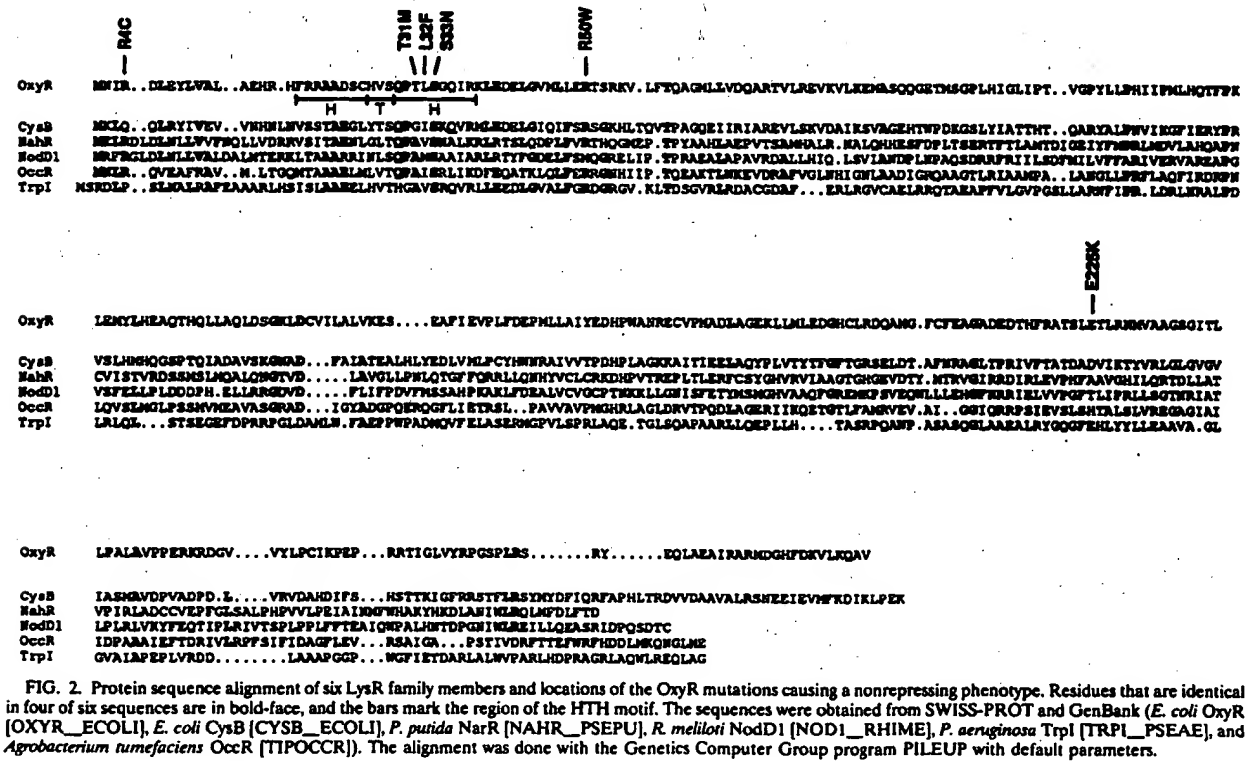

FIG. 2. Protein sequence alignment of six LysR family members and locations of the OxyR mutations causing a nonrepressing phenotype. Residues that are identical in four of six sequences are in bold-face, and the bars mark the region of the HTH motif. The sequences were obtained from SWISS-PROT and GenBank (*E. coli* OxyR [OXYR_ECOLI], *E. coli* CysB [CYSB_ECOLI], *P. putida* NarR [NAHR_PSEPU], *R. meliloti* NodD1 [NOD1_RHIME], *P. aeruginosa* TrpI [TRPI_PSEAE], and *Agrobacterium tumefaciens* OccR [TIPOCCR]). The alignment was done with the Genetics Computer Group program PILEUP with default parameters.

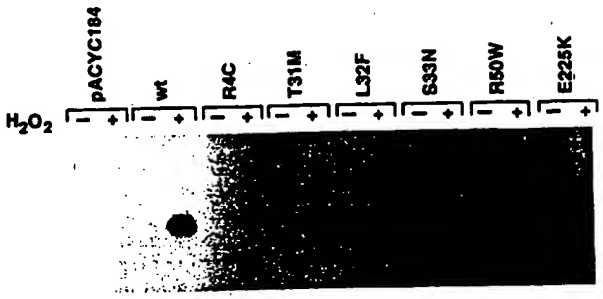

FIG. 3. Primer extension assays of *oxyS* induction in nonrepressing OxyR mutants. Exponential-phase cultures expressing the indicated OxyR mutants were split, and half of each culture was treated with 200 μM hydrogen peroxide for 10 min. Total RNA was then isolated from the treated (+) and untreated (−) cells, and a labeled oligonucleotide capable of hybridizing to the *oxyS* transcript was incubated with 3 μg of each RNA sample and extended with reverse transcriptase. wt, wild type.

of these mutants to induce *oxyS* expression were reduced to 5 to 15% of the wild-type activity. The S33N mutant was also assayed for its effect on the expression of the OxyR-regulated *ahpC* and *dps* genes, but as with *oxyS*, no transcriptional activation was detected (data not shown).

DNA binding by nonrepressing mutants. We next examined the DNA binding properties of the nonrepressing mutants by DNase I footprinting and DNA mobility shift assays. Extracts from strains carrying a chromosomal deletion of *oxyR* and either the wild-type or the mutant *oxyR* genes on pACYC184 were assayed for binding to an *oxyR-oxyS* promoter fragment. No DNase I footprint was observed for the nonrepressing

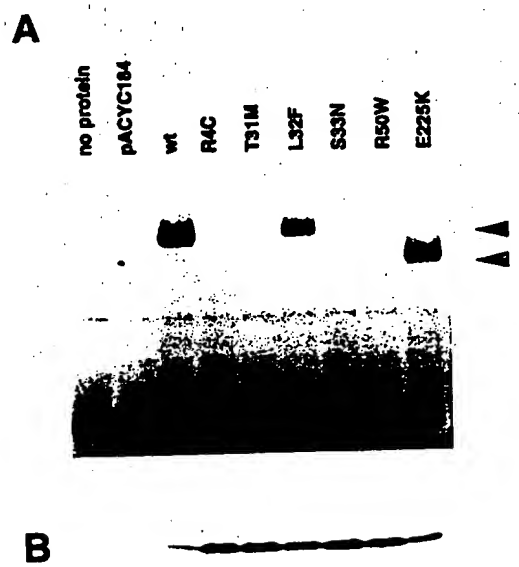

FIG. 4. (A) Gel retardation assay of nonrepressing OxyR mutants binding to the *oxyR-oxyS* promoters. Equal aliquots (1 μl) of the extracts were incubated with a 100-bp *Eco*RI-*Hin*dIII fragment of pGSO40 (37) labeled at the *Hin*dIII site. The bound and unbound fragments were then separated on a low-ionic-strength polyacrylamide gel. The filled and stippled arrows indicate two different protein-DNA complexes formed. (B) Immunoblot of the extracts used in the gel retardation assay. Equal aliquots of the crude extracts were loaded in each lane. wt, wild type.

FIG. 5. Gel retardation assay of purified wild-type (wt) and A233V and E225K mutant proteins. binding to the *oxyR-oxyS* promoter. Eight nanograms of the purified wild-type protein and 800 ng of the purified A233V and E225K mutant proteins were assayed. The filled and stippled arrows indicate the two different protein-DNA complexes observed.

mutants (Fig. 4 in reference 21 and data not shown). However, when DNA binding was assayed in a more sensitive gel retardation experiment, two of the mutants (L32F and E225K) showed a retarded band (Fig. 4A). The immunoblot in Fig. 4B shows that a full-length OxyR protein was present in all of the mutant extracts but that the mutant strains expressed higher levels of OxyR protein than the wild-type strain, in agreement with the nonrepressing phenotype of the mutants. Since equal amounts of total protein were assayed for all of the strains and the wild-type strain expressed significantly lower levels of OxyR, the relative binding affinity of the nonrepressing mutants is even lower than indicated by the intensity of the shifted band. The weak binding observed with mutants L32F and E225K correlates well with the weak transcriptional activity seen for these mutants in vivo. Only mutant R4C did not show any detectable binding to DNA but could slightly activate *oxyS* expression.

Interestingly, the E225K mutant led to a faster-migrating protein-DNA complex than the wild-type strain and mutant L32F. We had observed a similar faster-migrating complex in gel retardation experiments with extracts of a strain expressing the constitutively active A233V mutant described in the accompanying paper (21). To study the E225K and A233V proteins in vitro, the nonrepressing and constitutively active mutants were overexpressed and purified (21). When the gel retardation experiment was repeated with the pure proteins, it was obvious that both mutants showed protein-DNA complexes which had identical mobilities but migrated faster than the wild-type OxyR-DNA complex (Fig. 5). Both mutants also had significantly lower apparent affinities to the DNA than the wild-type protein, since a much larger amount of the pr tein (about 100-fold) was needed to obtain a visible retarded band. The increased mobility of the mutant protein-DNA complexes was not a function of the elevated protein concentrations needed to see binding, since the mobility of the wild-type OxyR-DNA complex was not altered by higher concentrations of wild-type protein (data not shown).

Oligomerization states of wild-type OxyR and A233V, E225K, and C199S mutants. Since the altered mobility ob-

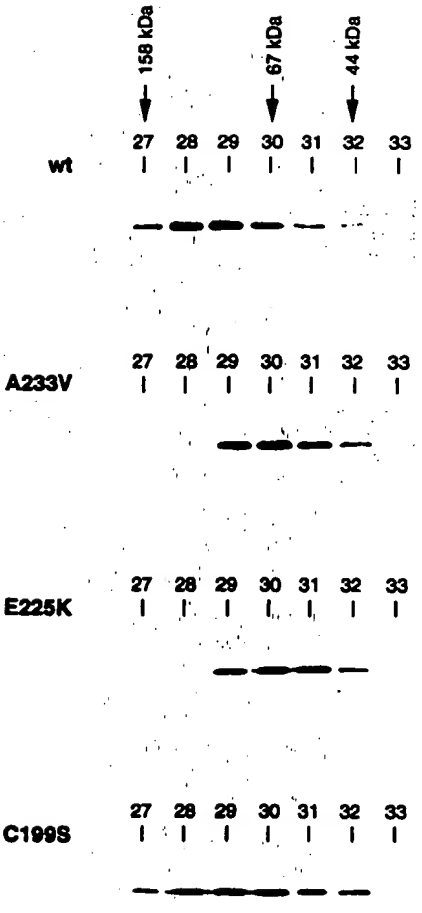

FIG. 6. Gel filtration analysis of wild-type (wt) OxyR and A233V, E225K, and C199S mutants. The purified proteins were run on a Superose 12 column, and the fractions were analyzed by immunoblots. The numbers denote the fraction numbers. The elution positions of the peak fractions for the standard proteins gamma globulin (158 kDa), bovine serum albumin (67 kDa), and ovalbumin (44 kDa) are indicated by the arrows.

served with the A233K and E225K protein-DNA complexes could be due to a difference in oligomerization, we examined the properties of the purified wild-type and A233V and E225K mutant proteins on a gel filtration column. Previous cross-linking experiments suggested that OxyR is a dimer in solution (35); however, several observations about the OxyR binding sites, such as the twofold dyad symmetry of the OxyR binding motif and the contacts made by OxyR in four adjacent major grooves, suggested that OxyR may act as a tetramer (37). The purified wild-type, A233V, and E225K mutant proteins were loaded on a Superose 12 gel filtration column, and the fractions were analyzed by immunoblots (Fig. 6). The wild-type protein eluted predominantly in fractions 28 to 30, with a peak between fractions 28 and 29. Assuming a globular conformation, this elution profile could correspond to a tetramer of the 34.4-kDa OxyR protein (137.6 kDa). The A233V and E225K mutant proteins eluted in fractions 29 to 31 with a peak at fraction 30, coinciding with the peak for the 67-kDa standard protein and suggesting that the A233V and E225K proteins are

TABLE 3. Galactokinase activities of *oxyR*$^+$ wild-type (GSO27) and Δ*oxyR*::*kan* deletion (GSO7) strains encoding an *oxyR-galK* fusion and carrying the nonrepressing mutants on pACYC184

Protein	*galK* activity (U)[a]		Fold derepression[b]
	oxyR$^+$ strain	Δ*oxyR*::*kan* strain	
Vector	0.8 ± 0.4	6.9 ± 1.6	1
Wild type	0.6 ± 0.1	0.5 ± 0.5	0.8
R4C	5.7 ± 1.7	5.7 ± 1.0	7.1
T31M	6.9 ± 0.4	6.8 ± 0.7	8.6
L32F	4.3 ± 1.1	5.7 ± 0.1	5.4
S33N	5.2 ± 1.7	5.7 ± 0.2	6.5
R50W	4.1 ± 1.5	7.8 ± 1.2	5.1
E225K	1.1 ± 0.8	3.5 ± 0.2	1.4

[a] The units of galactokinase activity were calculated as described previously (40). The average from two separate assays is given with the standard deviation.
[b] The fold derepression was calculated by dividing the units of *galK* activity in the *oxyR*$^+$ strains carrying the *oxyR* mutants by the units of activity in the *oxyR*$^+$ strain carrying the vector (0.8 U).

dimers (68.8 kDa). These data are consistent with the observation that the A233V and E225K mutants led to a faster-migrating protein-DNA complex in the gel retardation assay and may bind to DNA as dimers.

Since wild-type OxyR purified in the absence of reducing agents is oxidized, we also examined the size of the wild-type protein when the sizing column was loaded and eluted in the presence of 100 mM dithiothreitol. The elution profile observed under reducing conditions was almost identical to the profile seen in the absence of dithiothreitol (data not shown). We also examined the size of the noninducible C199S mutant, which appears to be locked in the reduced conformation (21). The C199S protein eluted over a greater range of fractions than the wild-type protein, but much of the protein appears to be tetrameric. Therefore, since both oxidized and reduced wild-type OxyR can be described as tetramers and both the tetrameric wild-type protein and the dimeric E225K mutant are sensitive to oxidation, our results suggest that oxidation of the OxyR protein does not influence multimerization.

***trans* dominance of nonrepressing mutants.** Having observed that OxyR is oligomeric, we tested whether the nonrepressing mutant proteins exhibit a negative *trans*-dominant phenotype and could inhibit the activity of the wild-type protein by forming inactive heteromultimers in vivo. We therefore transformed the plasmids encoding the nonrepressing mutants into a wild-type *oxyR* strain carrying the *oxyR-galK* fusion (GSO27) and compared the levels of *galK* expression with that in the original Δ*oxyR*::*kan* deletion (GSO7) background. As seen during the mutant screen, the vector-control strain and all of the nonrepressing mutants gave rise to red colonies in the Δ*oxyR*::*kan* background. In the *oxyR*$^+$ background, the vector-control strain gave white colonies since the chromosomally encoded OxyR protein could repress the *oxyR-galK* fusion. In contrast, the colonies for the R4C, T31M, L32F, S33N, and R50W mutants were red, showing that the chromosomally encoded wild-type protein could not repress the fusion in the presence of these mutants. The colonies for the *oxyR*$^+$ strain carrying E225K, however, were white, suggesting that the wild-type protein can still act as a repressor in the presence of this mutant.

We then assayed the levels of galactokinase activity in the *oxyR*$^+$ and Δ*oxyR*::*kan* strains in a quantitative assay (Table 3). In the Δ*oxyR*::*kan* deletion background, as expected, all of the mutants had levels of galactokinase activity comparable to that of the vector-control strain. In the *oxyR*$^+$ background, all mu-

tants except E225K had elevated levels of galact kinase activity and showed a nonrepressing phenotype. Therefore, the R4C, T31M, L32F, S33N, and R50W mutants are *trans* dominant and are able to "poison" the wild-type activity. The wild-type protein could still repress *oxyR* expression in the E225K mutant strain, suggesting that no mixed, inactive multimers are formed with E225K and revealing that this mutant is not *trans* dominant. This observation is consistent with our conclusion that the E225K mutation affects the multimerization of OxyR.

DISCUSSION

OxyR is a specific-DNA-binding protein which is able to activate as well as repress transcription of specific target genes. Here we used random mutagenesis to define regions of OxyR involved in DNA binding. We screened for mutants unable to repress an *oxyR-galK* fusion and identified six mutants which had elevated levels of *oxyR-galK* expression and exhibited decreased DNA binding in mobility shift and DNase I footprinting assays. The decreased-binding mutants also showed increased sensitivity to oxidants and decreased expression of the OxyR-activated *oxyS* gene in vivo, showing that OxyR-DNA binding is required for both activation and repression of the target genes.

The HTH motif represents a DNA binding domain of OxyR. Five of the six mutations (R4C, T31M, L32F, S33N, and R50W) mapped within or near the HTH motif which is conserved among the LysR family members. The decreased-binding phenotype of the nonrepressing mutants is consistent with the conclusion that the HTH region (residues 6 to 66) corresponds to the DNA binding domain in the LysR-type proteins (28). The R4C, S33N, and R50W mutations in OxyR affect amino acids that show a high degree of conservation among the LysR family members. Since many LysR family members bind to sequences that contain the very generic T-N_{11}-A motif (18), we propose that some of the highly conserved amino acids contact these conserved base pairs, while contacts by less highly conserved amino acids in the HTH domain provide specificity for the individual regulators. Future cross-linking experiments between the LysR-type proteins and the corresponding binding sites could test this hypothesis.

For OxyR, a region near the C terminus may also be critical for binding, since a protein with 22 amino acids truncated was not able to repress OxyR expression. A deletion of the C-terminal eight amino acids of the NahR protein also results in a loss of DNA binding (29). In contrast, the MetR protein tolerates substantial C-terminal deletions (38), suggesting that the C terminus is critical for DNA binding by only a subclass of LysR family members.

A C-terminal region of OxyR involved in tetramerization. One mutation (E225K) causing the nonrepressing phenotype did not map to the HTH motif but still caused decreased DNA binding. Since both the nonrepressing E225K mutant and the constitutively active A233V mutant led to faster-migrating protein-DNA complexes than wild-type OxyR in a gel retardation assay, we examined the sizes of these mutant proteins on gel filtration columns. While the oxidized wild-type OxyR protein was primarily tetrameric in solution, the E225K and A233V mutants appeared to be dimeric. This observation indicates that the E225K and A233V mutants are defective in multimerization and that the amino acids around positions 225 to 233 are directly or indirectly involved in tetramerization. Consistent with this interpretation, we found that the E225K mutant, unlike the other nonrepressing mutants, was not *trans* dominant.

The purified E225K and A233V mutants are similar in their abilities to bind DNA and induce expression f *oxyS* in vitro (21); however, several in viv characteristics of these mutants are distinct. Both mutant proteins show weak binding in vitro, but E225K does n t repress *oxyR* expression in viv , while A233V is an efficient repressor. The E225K protein was also only a weak activator f *oxyS* expressi n under oxidizing conditions in vivo, while the A233V mutant was an extremely strong constitutive activator. Since the purified A233V protein had only a weak activity in vitro compared with its strong activation activity in vivo, it is likely that the A233V protein is modified during purification. Possibly some unknown conditions or factors present in intact cells act to stabilize the A233V mutant.

Our observation that the oxidized wild-type OxyR protein is a tetramer is consistent with the previous finding that the OxyR protein binds to four adjacent major grooves of the DNA helix (37). The twofold dyad symmetry of the OxyR binding m tif (ATAGxtxxxaxCTATxxxxxxxATAGxtxxxaxCTAT) suggests that the tetrameric OxyR protein may exist as a dim r of dimers (37). This would imply the presence of two multimerization domains in OxyR, one involved in dimerization and the other required for the tetramerization of two dimers. The E225K and A233V mutations could conceivably affect either the dimerization domain (resulting in dimers via the tetramerization domain) or the tetramerization domain (resulting in dimers via the dimerization domain). We cannot unambiguously distinguish between these possiblities, but we propose that the two OxyR mutations affect a putative tetramerizati n domain, since the C terminus seems to be dispensible f r dimeric but not tetrameric LysR family members. We did not identify any mutations affecting the second multimerizati n domain. Possibly these mutations lead to an unstable form of the protein, or alternatively, the mutants are still *trans* dominant.

The NahR, TrpI, and CysB members of the LysR family have also been shown to be tetramers in solution (14, 26, 29), while other LysR proteins, such as MetR, NodD3, and Nac, appear to be dimers (17, 19, 25). The CysB protein has also recently been shown to bind DNA as a tetramer (20). It is interesting that for OxyR and possibly for other tetrameric LysR family members, the C-terminal domain is critical for DNA binding and the protein binds to approximately 45 bp. In contrast, for dimeric MetR, the C terminus seems to be dispensable for DNA binding and the protein binds to approximately 25 bp (reference 24 and references therein; 38). These differences suggest that there may be at least two different classes of LysR proteins, those which are able to bind and activate as dimers and others which are able to function only as tetramers.

The finding that OxyR exists as a multimer raises the possibility that the oligomerization state of OxyR might be regulated as a function of oxidation and reduction, similar to the case for the oxygen-sensitive transcriptional regulator FNR (23). We do not favor this mechanism for regulating the activity of OxyR, since the elution profile of wild-type OxyR under reducing conditions (data not shown) is identical to the elution profile of the oxidized protein. The inactive C199S mutant, which appears to be locked in the reduced state (21) and shows a protein-DNA shift similar to that with the oxidized wild-type protein (data not shown), can also be described as a tetramer. In addition, the dimeric A233V mutant is constitutively active, and the dimeric E225K protein is still responsive to hydr gen peroxide in vivo. Future studies of how the OxyR subunits are arranged, how the subunit contacts are changed upon oxidation, and how many subunits need to be oxidized in order to

induce transcripti n sh uld give further insights into how the OxyR protein is activated by oxidation.

ACKNOWLEDGMENTS

We thank S. Adhya for strains and plasmids, S. Garges for assistance with the galactokinase assays, and S. Altuvia, S. Garges, R. Klausner, and C. Wu for helpful discussions and comments on the manuscript.

I.K. was the recipient of fellowships from the Schweizerischen Nationalfonds and the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Adhya, S.** Unpublished data.
2. **Adhya, S., and W. Miller.** 1979. Modulation of the two promoters of the galactose operon of *Escherichia coli*. Nature (London) 279:492–494.
3. **Altuvia, S., M. Almirón, G. Huisman, R. Kolter, and G. Storz.** 1994. The *dps* promoter is activated by OxyR during growth and by IHF and σ^S in stationary phase. Mol. Microbiol. 13:265–272.
4. **Altuvia, S., and G. Storz.** Unpublished data.
5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley and Sons, New York.
6. **Bartowsky, E., and S. Normark.** 1991. Purification and mutant analysis of *Citrobacter freundii* AmpR, the regulator for chromosomal AmpC β-lactamase. Mol. Microbiol. 5:1715–1725.
7. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. 68:245–267.
8. **Bölker, M., and R. Kahmann.** 1989. The *Escherichia coli* regulatory protein OxyR discriminates between methylated and unmethylated states of the phage Mu *mom* promoter. EMBO J. 8:2403–2410.
9. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA 81:6929–6933.
10. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques 5:376.
11. **Burn, J. E., W. D. Hamilton, J. C. Wootton, and A. W. B. Johnston.** 1989. Single and multiple mutations affecting properties of the regulatory gene *nodD* of *Rhizobium*. Mol. Microbiol. 3:1567–1577.
12. **Calos, M. P.** 1978. DNA sequence for a low-level promoter of the *lac* repressor gene and an "up" promoter mutation. Nature (London) 274:762–765.
13. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141–1156.
14. **Chang, M., and I. P. Crawford.** 1991. In vitro determination of the effect of indoleglycerol phosphate on the interaction of purified TrpI protein with its DNA-binding sites. J. Bacteriol. 173:1590–1597.
15. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell 41:753–762.
16. **Christman, M. F., G. Storz, and B. N. Ames.** 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA 86:3484–3488.
17. **Fisher, R. F., and S. R. Long.** 1993. Interactions of NodD at the *nod*-box: NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. J. Mol. Biol. 233:336–348.
18. **Goethals, K., M. Van Montagu, and M. Holsters.** 1992. Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 reveal a common structure in promoters regulated by LysR-type proteins. Proc. Natl. Acad. Sci. USA 89:1646–1650.
19. **Goss, T. J., and R. A. Bender.** Unpublished data.
20. **Hryniewicz, M. M., and N. M. Kredich.** 1994. Stoichiometry of binding of CysB to the *cysJIH*, *cysK*, and *cysP* promoter regions of *Salmonella typhimurium*. J. Bacteriol. 176:3673–3682.
21. **Kullik, I., M. B. Toledano, L. A. Tartaglia, and G. Storz.** 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. J. Bacteriol. 177:1275–1284.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
23. **Lazazzera, B. A., D. M. Bates, and P. J. Kiley.** 1993. The activity of the *Escherichia coli* transcription factor FNR is regulated by a change in oligomeric state. Genes Dev. 7:1993–2005.
24. **Mares, R., M. L. Urbanowski, and G. V. Stauffer.** 1992. Regulation of the *Salmonella typhimurium metA* gene by the MetR protein and homocysteine. J. Bacteriol. 174:390–397.
25. **Maxon, M. E., J. Wigboldus, N. Brot, and H. Weissbach.** 1990. Structure-function studies on *Escherichia coli* MetR protein, a putative prokaryotic leucine zipper protein. Proc. Natl. Acad. Sci. USA 87:7076–7079.
26. **Miller, B., and N. M. Kredich.** 1987. Purification of the *cysB* protein from *Salmonella typhimurium*. J. Biol. Chem. 262:6006–6009.
27. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. 47:597–626.
29. **Schell, M. A., P. H. Brown, and S. Raju.** 1990. Use of saturation mutagenesis to localize probable functional domains in the NahR protein, a LysR-type transcription activator. J. Biol. Chem. 265:3844–3850.
30. **Storz, G., and S. Altuvia.** 1994. OxyR regulon. Methods Enzymol. 234:217–223.
31. **Storz, G., M. F. Christman, H. Sies, and B. N. Ames.** 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA 84:8917–8921.
32. **Storz, G., L. A. Tartaglia, and B. N. Ames.** 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science 248:189–194.
33. **Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa.** 1989. Molecular cloning and nucleotide sequencing of *oxyR*, the positive regulatory gene of a regulon for an adaptive response to oxidative stress in *Escherichia coli*: homologies between OxyR protein and a family of bacterial activator proteins. Mol. Gen. Genet. 218:371–376.
34. **Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa.** 1991. Purification and characterization of the *Escherichia coli* OxyR protein, the positive regulator for a hydrogen peroxide-inducible regulon. J. Biochem. 109:262–266.
35. **Tartaglia, L. A., C. J. Gimeno, G. Storz, and B. N. Ames.** 1992. Multidegenerate DNA recognition by the OxyR transcriptional regulator. J. Biol. Chem. 267:2038–2045.
36. **Tartaglia, L. A., G. Storz, and B. N. Ames.** 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. J. Mol. Biol. 210:709–719.
37. **Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell 78:897–909.
38. **Urbanowski, M. L., and G. V. Stauffer.** Unpublished data.
39. **Warne, S. R., J. M. Varley, G. J. Boulnois, and M. G. Norton.** 1990. Identification and characterization of a gene that controls colony morphology and auto-aggregation in *Escherichia coli* K12. J. Gen. Microbiol. 136:455–462.
40. **Wilson, D. B., and D. S. Hogness.** 1966. Galactokinase and uridine diphosphogalactose 4-epimerase from *Escherichia coli*. Methods Enzymol. 8:229–240.

MECHANISMS IN THE PATHOGENESIS OF ENTERIC DISEASES

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PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging-in-Publication Data

Mechanisms in the pathogenesis of enteric diseases / edited by Prem S. Paul, David H. Francis, and David A. Benfield.
p. cm. -- (Advances in experimental medicine and biology ; v. 412)
"Proceedings of the First International Rushmore Conference on Mechanisms in the Pathogenesis of Enteric Diseases, held September 28-30, 1995, in Rapid City, South Dakota"--T.p. verso.
Includes bibliographical references and index.
ISBN 0-306-45519-6
1. Infection--Pathogenesis--Congresses. 2. Communicable diseases--Pathogenesis--Congresses. I. Paul, Prem S. II. Francis, David H. III. Benfield, David A. IV. International Rushmore Conference on Mechanisms in the Pathogenesis of Enteric Diseases (1st : 1995 : Rapid City, S.D.) V. Series.
[DNLM: 1. Gastrointestinal Diseases--physiopathology--congresses. 2. Gastrointestinal Diseases--etiology--congresses. W1 AD559 v.412 1997 / WI 140 M486 1997]
RB153.M43 1997
616.3'307--dc21
DNLM/DLC
for Library of Congress

97-5561
CIP

Proceedings of the First International Rushmore Conference on Mechanisms in the Pathogenesis of Enteric Diseases, held September 28 – 30, 1995, in Rapid City, South Dakota

ISBN 0-306-45519-6

A Division of Plenum Publishing Corporation
233 Spring Street, New York, N. Y. 10013

http://www.plenum.com

10 9 8 7 6 5 4 3 2 1

Printed in the United States of America

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A NOVEL REGULATORY MECHANISM FOR A NOVEL PHASE-VARIABLE OUTER MEMBRANE PROTEIN OF *ESCHERICHIA COLI*

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SUMMARY

Antigen 43 (Ag43) is a prominent hetero-oligomeric protein complex in the outer membrane of *Escherichia coli*. It is composed of two subunits. α^{43} (M_r 60, 000) and β^{43} (M_r 53, 000) in 1:1 stoichiometry. α^{43} is surface expressed, extends beyond the O-side chains of smooth lipopolysaccharide and is bound to the cell surface through an interaction with β^{43}, itself an integral outer membrane protein. α^{43} shows limited sequence homology with some enterobacterial adhesins. Expression of Ag43 is subject to reversible phase variation, the rates of variation from the Ag43+ve to Ag43-ve states in liquid minimal medium being ~2.2 x 10^{-3}, the corresponding rates from Ag43-ve to Ag43+ve states being ~1 x 10^{-3}. Phase switching of genes encoding Ag43 are transcriptionally regulated by DNA methylation (deoxyadenosine methylase [*dam*] mutants being "locked OFF") and by OxyR (*oxyR* mutants being "locked ON"). It is proposed that OxyR acts as a repressor of Ag43 transcription by binding to unmethylated GATC sites in the regulatory region of the gene. Sequencing and mapping has identified Ag43 as the likely product of the metastable *flu* gene first described in 1980 by Diderichsen and responsible for colony form variation in *E. coli*.

INTRODUCTION

A number of years ago, workers in this laboratory identified a prominent new protein antigen (termed Ag43) in the outer membrane of *Escherichia coli*. An *Escherichia*-specific antigen found in almost all *E. coli* strains, Ag43 is composed of two chemically and immunologically distinct protein subunits (termed α^{43} and β^{43}) present in 1:1 stoichiometry i.e. $(\alpha^{43}: \beta^{43})_x$. The α^{43} subunit has an apparent M_r of 60, 000. The other

Mechanisms in the Pathogenesis of Enteric Diseases, edited by Paul *et al.*
Plenum Press, New York, 1997

subunit is heat modifiable in a manner similar to that of several other outer membrane proteins (notably OmpA), and migrates with apparent M_rs of 37, 000 (β') if heated to temperatures of 70°C or below prior to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and 53, 000 (β) if heated at higher temperatures. The α^{43} and β^{43} polypeptides are not linked by disulphide bonds, contain no detectable carbohydrate, identifiable cofactors, acyl groups or enzyme activity (Owen, 1986; Owen et al., 1987).

The fact that the two subunits form a complex *in situ* is supported by (a), reconstitution experiments in which an α^{43}: β^{43} complex can be reformed from mixtures containing purified α^{43} and α^{43}-stripped membranes bearing only the β^{43} subunit (Caffrey and Owen, 1989; Owen et al., 1987); (b) the detection by SDS-PAGE and Western immunoblotting of a relatively unstable α43: β^{43} complex (M_r 110, 000 - 120, 000) capable of dissociating to give α^{43} and β^{43} monomers (Caffrey, 1988; Meehan, 1994); and (c) cross-linking experiments, performed using the homobifunctional reagents dimethylsuberimidate and dithio-*bis*(succinimidylproprionate), which clearly demonstrate the presence of α^{43}-α^{43}, and α^{43}-β^{43} interactions. There is also strong evidence from cross-linking experiments that Ag43 is in close proximity to the ferric-enterochelin receptor, FepA (Meehan, 1994; Meehan et al., 1992; Owen, 1992).

Careful fractionation experiments reveal that Ag43 is located exclusively in the outer membrane (Owen, 1985; Owen et al., 1987). Comprehensive progressive immunoadsorption experiments, immunofluorescence studies, limited proteolysis and immunogold labelling experiments performed on thin sections of *E. coli* (Caffrey, 1988; Meehan, 1994; Meehan et al., 1992; Owen, 1983; Owen, 1992; Owen et al., 1987), together strongly support a working model in which the α^{43} subunit is a peripheral protein expressed on the surface of the outer membrane and anchored to it by virtue of specific interactions with β^{43}, such protein-protein interactions effectively protecting the C-terminus of α^{43} from proteolysis. α^{43} penetrates the O-antigen side chains of smooth lipopolysaccharide (S-LPS) and is a major surface antigen. β^{43}, an integral membrane protein resides closer to the membrane surface, its determinants only becoming unmasked in strains bearing truncated rough (R)-LPS molecules.

Ag43 shows some properties in common with fimbriae. The α^{43} (but not the β^{43}) subunit can be selectively and almost quantitatively released from *E. coli* outer membranes by brief heating to 60°C, thereby providing an effective means of purification (Caffrey and Owen, 1989). Furthermore, the N-terminal amino acid sequence of α^{43} contains a stretch of six residues (TVNGGT) which is also present in the N-termini of the major subunits of several enterobacterial fimbriae (Fig. 1) (Caffrey and Owen, 1989; Owen et al., 1991). Of additional interest is the observation that V8 proteolytic cleavage of denatured α^{43} generates, amongst other species, three low-M_r peptides with distinct N-terminal amino acid sequences, each showing about 50% homology with internal sequences within AIDA-I (Henderson et al., 1994, 19955), an outer membrane protein adhesin of diffuse adhering *E. coli* (Benz and Schmidt, 1992). However, electron microscopic studies of negatively stained preparations of cells, purified outer membranes, free α^{43} or immunoprecipitated Ag43, and deep etching of whole cells has not revealed any morphological features recognisable as fimbriae or, indeed, any other regular repeating structures such as S-layers (Caffrey, 1988; Meehan, 1994; Owen et al., 1987; Owen et al., 1991). It should be noted, perhaps, that some such structures have proved exceptionally difficult to visualise in the electron microscope. Therefore, more sophisticated methodology may be required in order to appreciate the organisation assumed by Ag43 on the cell surface.

α^{43}	A	D	I	V	V	H	P	G	E	T	V	N	G	G	T	L	A	N
E. coli FM1A							A	A	T	T	V	N	G	G	T	V	H	F
E. coli FM1C							A	V	T	T	V	N	G	G	T	V	H	F
E. coli FM1F							D	T	T	T	V	N	G	G	T	V	H	F
E. coli S								V	T	T	V	N	G	G	T	V	H	F
E. coli S-II							A	P	A	T	V	N	G	G	T	V	H	F
E. coli F1C							A	V	T	T	V	N	G	G	T	V	H	F
E. coli NFA-1						D	A	N	G	L	T	V	N	A	G	D	G	K	N
K. pneumoniae FM11								D	T	T	T	V	N	G	G	T	V	H	F
K. pneumoniae FM12								D	T	T	T	V	N	G	G	T	V	H	F
P. mirabilis MR/P	T	G	T	P	A		P	T	E	T	V	D	G	G	T	I	D	F
S. typhimurium type 1						P	T	P	V	S	V	S	G	G	T	I	H	P
S. enteritidis SEF21						P	T	P	V	S	V	S	G	G	T	I	H	P

Figure 1. Comparison of the revised N-terminal sequence of α^{43} with the N-termini of various fimbrial subunits (data from Caffrey and Owen, 1989; Henderson et al., 1995 and GenBank Database). Amino acids are given in the standard single letter code. Identical amino acid residues are boxed.

AG43 UNDERGOES PHASE VARIATION

Ag43 can be shown to undergo reversible phase variation (Caffrey and Owen, 1989). Thus, Ag43+ve and Ag43-ve variants can be distinguished readily by colony immunoblotting using anti-Ag43 or subunit-specific antisera. Furthermore, SDS-PAGE analysis of outer membranes confirms the dramatically decreased levels of both α^{43} and β^{43} subunits in Ag43-ve variants.

Immunofluorescence experiments conducted with specific anti-α^{43} or anti-Ag43 sera confirm that 80–90% of cells derived from Ag43+ve colonies express Ag43 on their surface, whereas the remaining 10–20% are switched off for expression of Ag43. Conversely, only a minority (1–5%) of cells derived from Ag43-ve colonies express Ag43. Screening of progeny derived from both positive and negative variants grown in succinate minimal media indicates that the rates of phase variation from Ag43+ve to Ag43-ve states and from Ag43-ve to Ag43+ve states approximates to 2.2×10^{-3} and 1×10^{-3}, respectively. Growth in rich media or at low temperature (24°C) causes a decrease in Ag43 expression. However, growth on glucose, in high osmolarity or in the presence of high or low Fe^{2+} does not dramatically affect either switching frequency or levels of expression (Meehan, 1994; Meehan et al., 1991).

REGULATION OF AG43 EXPRESSION

In bacteria, phase variation is generally controlled at two levels viz., at a local level by regulatory proteins encoded by the specific operon in question and, at a higher level, by global regulators, often DNA-binding proteins (Dorman, 1994). In an attempt to understand the regulation of Ag43 expression, a panel of well defined regulatory mutants (*lrp, crp, cya, recA, hns, gyrA, hu1, hu2, topA, rpoS, fis,* and *himA*) were examined by colony and Western immunoblotting and by immunofluorescence microscopy. All showed levels

and patterns of expression of Ag43 analogous to the wild type. Nor did the addition of leucine or alanine to the growth medium affect Ag43 expression in wild-type strains (the same amino acids caused a 32–16 fold reduction in K99 expression in *E. coli* 09 K[A]). This observation further serves to eliminate a role for Lrp in the regulation of Ag43. Notably, however, deoxyadenosine methylase (Dam) mutants of *E. coli* and transductants carrying the *dam* mutation totally lack Ag43 as judged by colony and Western immunoblotting and by immunofluorescence microscopy, i.e. all cells are "locked OFF" for Ag43 expression (Henderson et al., 1995; Henderson and Owen, 1994; Meehan, 1994). It should be recalled that Dam usually methylates the N6 position of adenine at all GATC sites in *E. coli*. The presence of unmethylated sites, particularly during replication, can be used by certain cellular processes in regulation (Messer and Noyer-weider, 1988). An example is the Pap operon (encoding P-fimbriae) in which Lrp competes with Dam for unmethylated GATC sites (Van der Woude et al., 1992).

OxyR mutants and transductants carrying *oxyR* mutations also show no evidence of phase switch. However, in contradistinction to *dam* mutants, *oxyR* mutants are firmly "locked ON" for Ag43 expression as judged by immunofluorescence microscopy and by Western and colony immunoblotting (Henderson et al., 1995). OxyR is a LysR-type transcriptional activator known to regulate expression of proteins important in oxidative stress. In these situations, OxyR is thought to actively bind RNA polymerase. There is at least one known example of OxyR acting as a transcriptional repressor viz. for the *mom* gene of phage Mu (Dorman, 1994; Kullik et al., 1995). Interestingly, introduction of the *dam* mutation into the *E. coli oxyR* mutant BD1302 fails to affect the constitutive "locked ON" expression of Ag43. The simplest model to explain the above is one in which OxyR acts as a repressor of Ag43 transcription by binding to unmethylated GATC sites in the regulatory region of the gene, thus preventing RNA polymerase from binding (Fig. 2) (Henderson et al., 1995).

AG43 IS THE *FLU* GENE PRODUCT

Using a set of nested primers designed from a knowledge of the N-terminal sequences of native α^{43} and a C-terminal proteolysis product, a 1.2kb-PCR product has recently been cloned and sequenced (Henderson et al., 1995). This PCR product corresponds to the bulk of the α^{43} gene and hybridises (Henderson et al., 1995) with two overlapping clones in the Kohara gene bank (Kohara et al., 1987). Significantly, these clones cover part of min 43 on the *E. coli* chromosome, a region which contains (at min 43.6) the metastable *flu* gene responsible for colony form variation in *E. coli*. Although first described in 1980 (Diderichsen, 1980), the *flu* gene has not been cloned nor has its gene product been identified. Despite this, *flu* continues to be widely cited in the literature as a prime example of phase variation in prokaryotes. Form 1 (Flu^-) variants are known to give rise to large, rough, irregular colonies and to autoaggregate in liquid media. Form 2 (Flu^+) variants, on the other hand, produce small, smooth colonies and do not autoaggregate (Diderichsen, 1980) (Fig. 3). Comprehensive analysis of authentic *E. coli flu* variants displaying predominantly Form 1 colonies (BD1512), predominantly Form 2 colonies (BD1511), and exclusively Form 1 colonies (*oxyR* mutant BD1302) by Western and colony immunoblotting and by immunofluorescence microscopy shows a direct and consistent correlation between the Flu^- phenotype and Ag43 expression (Henderson et al., 1995).

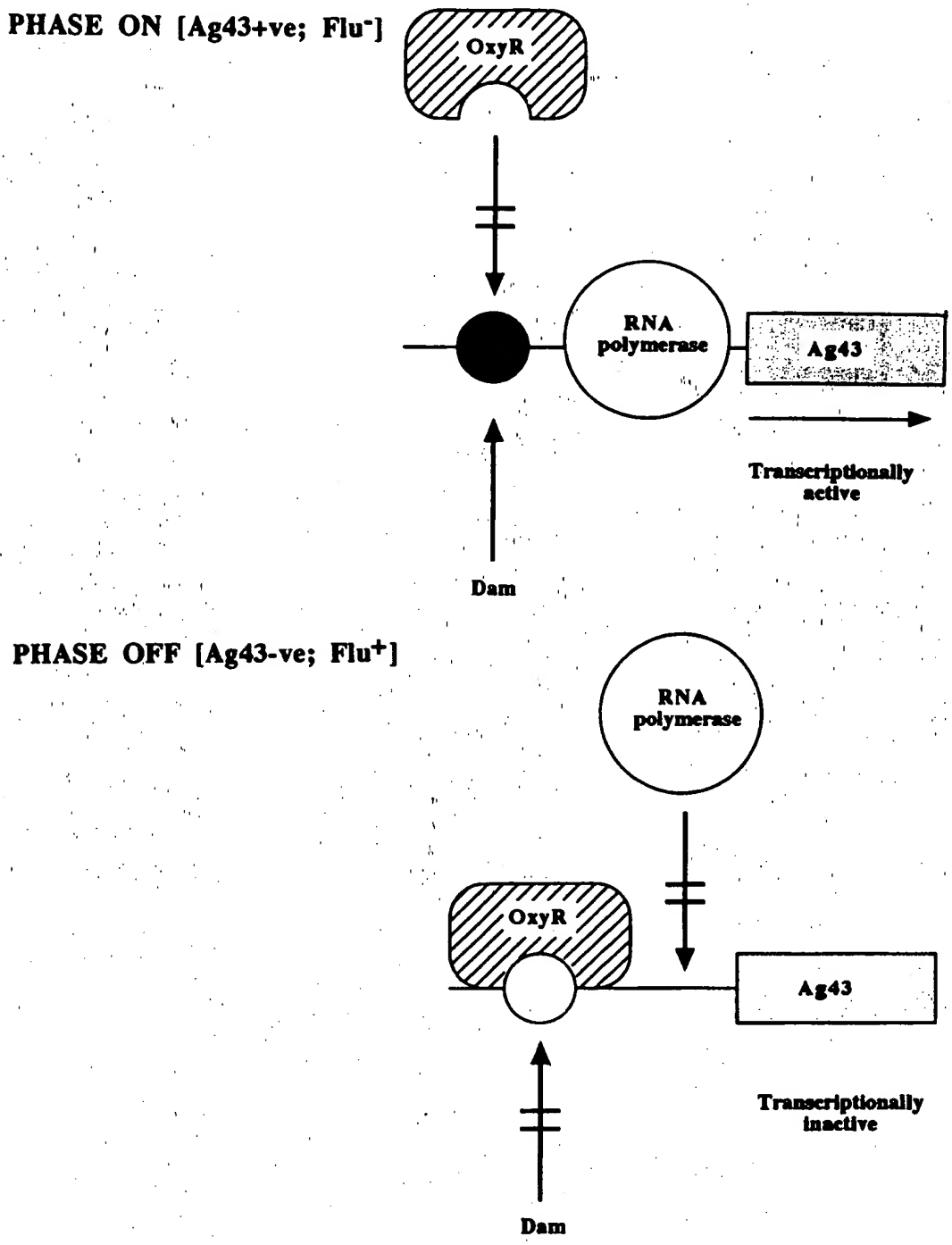

Figure 2. Working model for regulation of Ag43 expression. OxyR, Dam and RNA polymerase are shown interacting with their respective DNA binding sites. Methylated GATC sites are indicated by a solid circle and unmethylated GATC sites by an open circle. Hashed arrows indicate no binding. It is proposed that competition between OxyR and Dam for unmethylated GATC sites leads to phase variation. Methylation of GATC sites by Dam prevents OxyR binding and transcription proceeds (phase ON). Binding of OxyR to unmethylated GATC sites excludes RNA polymerase leading to the transcriptionally inactive (phase OFF) state. In *dam* mutants, *oxyR* permanently excludes RNA polymerase giving a "locked OFF" state. In *oxyR* mutants, RNA polymerase is not excluded leading to constitutive expression (i.e. "locked ON" state)

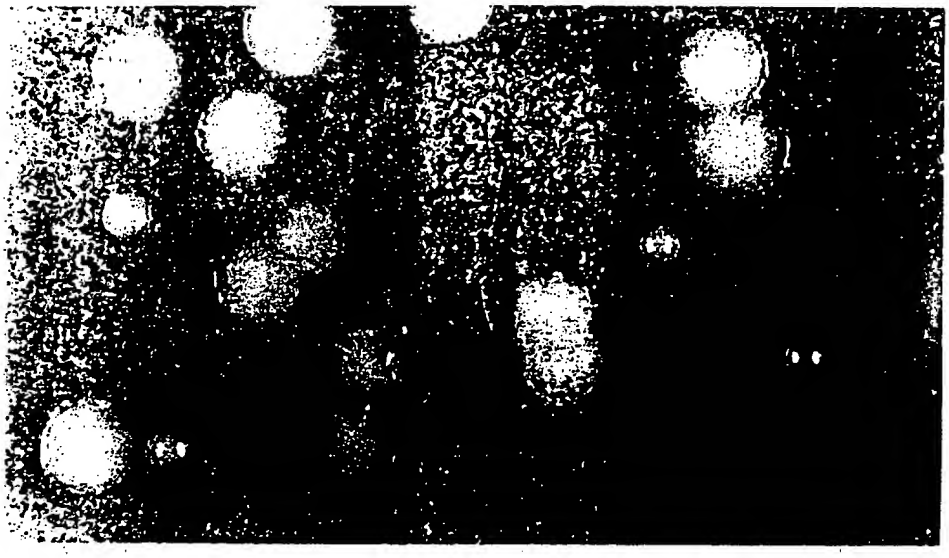

Figure 3. Colony form variation in *E. coli* BD1512. The large flat irregular colonies display the Flu⁻ phenotype. The majority of cells in such colonies express Ag43 and autoaggregate in liquid media. The small glossy smooth colonies display the Flu⁺ phenotype. The majority of cells from these colonies are non-autoaggregative, and do not express Ag43.

In conclusion, there seems little doubt that we have identified Ag43 as the elusive product of the metastable *flu* gene and have uncovered a novel regulatory mechanism governing its expression.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Health Research Board of Ireland. The authors would like to thank B. Diderichsen, C. Dorman and G. Storz for generous gifts of strains.

REFERENCES

Benz., I., and M. A. Schmidt. 1992. AIDA-I, the adhesin involved in diffuse adherence of the diarrhoeagenic *Escherichia coli* strain 2787 (0126:H27), is synthesized via a precursor molecule. Mol. Microbiol. 6:1539–1546.

Caffrey. P. 1988. Antigen 43 of the outer membrane of *Escherichia coli*. Ph.D. Thesis. Trinity College Dublin.

Caffrey. P., and P. Owen. 1989. Purification and N-terminal sequence of the α subunit of antigen 43, a unique protein complex associated with the outer membrane of *Escherichia coli*. J. Bacteriol. 171:3634–3640.

Diderichsen, B. 1980. *Flu*, a metastable gene controlling surface properties of *Escherichia coli*. J. Bacteriol. 141:858–867.

Dorman, C. 1994. Genetics of Bacterial Virulence. Blackwell Scientific Publications, Oxford.

Henderson, I., M. Meehan and P. Owen. 1995. Manuscript in preparation.

Henderson, I., M. Meehan, and P. Owen. 1994. Proteolytic studies on antigen 43 - a bipartite outer membrane protein of *Escherichia coli*, abstr. P-50, p. 52. Abstr. 129th Ord. Meet. Soc. Gen. Microbiol. 1994.

Henderson, I., and P. Owen. 1994. Dam methylation affects expression of antigen 43 in *Escherichia coli*, abstr. P-11. Abstr. Irish Branch Meet. Soc. Gen. Microbiol. (Jordanstown) 1994.

Kohara, Y., K. Akiyama, and K. Isono. 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell 50:495–508.

Kullik, I., M. B. Toledano, L. A. Tartaglia, and G. Storz. 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. J. Bacteriol. 177:1275–1284.

Meehan, M. 1994. Studies of antigen 43, a bipartite outer membrane protein of *Escherichia coli*. Ph.D. Thesis. Trinity College Dublin.

Meehan, M., P. Caffery, and P. Owen. 1991. Phase variation of an outer membrane protein of *Escherichia coli*, abstr. P8, p.45. Abstr. 119th Ord. Meet. Soc. Gen. Microbiol. 1991.

Meehan, M., H. Doherty, and P. Owen. 1992. Immunological studies of a bipartite outer membrane protein in *Escherichia coli*, abstr. P51, p.62. Abstr. 123rd Ord. Meet. Soc. Gen. Microbiol. 1992.

Messer, P., and M. Noyer-Weider. 1988. Timing and targeting: the biological functions of Dam methylation in *E. coli*. Cell 54:735–737

Owen, P. 1983. Antigens of the *Escherichia coli* cell envelope, p. 347–373. *In* O.J. Bjerrum (ed.), Electroimmunochemical analysis of membrane proteins. Elsevier Science Publishers B.V., Amsterdam.

Owen, P. 1985. Crossed immunoelectrophoresis in the study of outer membrane antigens, p. 207–272. *In* T. K. Korhonen, E. A. Dawes and P. H. Mäkelä (ed.), Enterobacterial surface antigens: methods for molecular characterisation. Elsevier Science Publishers BV., Amsterdam.

Owen, P. 1986. An improved procedure for polypeptide analysis of radiolabeled antigens resolved by crossed immunoelectrophoresis and its application to the study of inner and outer membranes of *Escherichia coli*. Electrophoresis. 7:19–28.

Owen, P. 1992. The Gram-negative outer membrane: structure, biochemistry and vaccine potential. Biochem. Soc. Trans. 20:1–6

Owen, P., P. Caffrey, and L.-G. Josefsson. 1987. Identification and partial characterization of a novel bipartite protein antigen associated with the outer membrane of *Escherichia coli*. J. Bacteriol. 169: 3770–3777.

Owen, P., P. Caffrey, L.-G. Josefsson and M. Meehan. 1991. Outer membrane proteins: old and new, p. 127–139. *In* E. Z. Ron and S. Rottem (ed.), Microbial surface components and toxins in relation to pathogenesis. Plenum Press, London.

Van der Woude, M. W., B. A. Braaten and D. A. Low. 1992. Evidence for global regulatory control of pilus expression in *Escherichia coli* by Lrp and DNA methylation: model building based on analysis of *pap*. Mol. Microbiol. 6:2429–2435.

11. Platinum bipolar stimulating electrodes were lowered 7 mm below the cortical surface 3.3 mm lateral and 2.3 mm posterior to the bregma in barbiturate-anesthetized rats weighing ~300 g and were cemented into place with the use of sterile techniques approved under animal care protocol of the University of California, San Francisco. After 2 weeks of recovery, 250-ms (or a 15-Hz train of six 25-ms) 50-dB sound pressure level tones were paired with 200 ms of NB electrical stimulation in a sound-shielded, calibrated test chamber (5 days per week). Electrical stimulation began either 50 ms after tone onset (n = 15) or 200 ms before (n = 6). The two timings did not appear to affect plasticity, and data from both groups were pooled. The current level (70 to 150 μA) was chosen to be the minimum necessary to desynchronize the EEG during slow-wave sleep for 1 to 2 s. Stimulation consisted of 100-Hz capacitatively coupled biphasic pulses of 0.1 ms duration. Tonal and electrical stimuli did not evoke any observable behavioral responses (that is, did not cause rats to stop grooming or if sleeping, awaken).
12. Twenty-four hours after the last pairing, animals were anesthetized with pentobarbital and the right auditory cortex was surgically exposed. Parylene-coated tungsten microelectrodes (2 megohms) were lowered 550 μm below the pial surface (layer 4/5), and complete tuning curves were generated with 50-ms pure tones (with 3-ms ramps) presented at 2 Hz to the contralateral ear. The evoked spikes of a small cluster of neurons were collected at each site. To determine the effects of conditioning on the bandwidth of individual neurons, spike waveforms were collected during eight experiments and were sorted offline with software from Brainwave Technologies. Penetration locations were referenced using the cortical vasculature as landmarks. The primary auditory cortex was defined on the basis of its short latency (8- to 20-ms) responses and continuous tonotopy (BF increases from posterior to anterior). Responsive sites that exhibited clearly discontinuous BFs and either long latency responses, an unusually high threshold, or very broad tuning were considered to be non-A1 sites. Penetration sites were chosen to avoid blood vessels while generating a detailed and evenly spaced map. The edges of the map were estimated with the use of a line connecting the nonresponsive and non-A1 sites. Map reorganizations resulted in significant effects on the outline of A1, although no particular pattern was observed. The effect of conditioning on mean bandwidths across all conditions was determined with analysis of variance; pairwise comparisons were analyzed by Bonferroni post-hoc tests.
13. The set of tone frequencies presented at each site was approximately centered on the BF of each site. Thus, during analysis each tuning curve was approximately centered in the stimulus space, and simply blanking the axes and analyzing the sites in random order allowed for tuning curve characterization to be completely blind. With the use of custom analysis software, the tuning curve edges for each site were defined by hand and recorded without the possibility of experimenter bias.
14. S. L. Sally and J. B. Kelly, *J. Neurophysiol.* 59, 1627 (1988).
15. G. H. Recanzone, C. E. Schreiner, M. M. Merzenich, *J. Neurosci.* 13, 87 (1993).
16. W. M. Jenkins, M. M. Merzenich, M. T. Ochs, T. Allard, E. Guic-Robles, *J. Neurophysiol.* 63, 82 (1990).
17. G. H. Recanzone, M. M. Merzenich, W. M. Jenkins, K. A. Grajski, H. R. Dinse, *Ibid.* 67, 1031 (1992).
18. M. P. Kilgard and M. M. Merzenich, unpublished observation.
19. In contrast to the large changes induced by pairing tones with NB stimulation, no significant cortical map reorganizations were observed in previous experiments after tens of thousands of behaviorally irrelevant stimuli were presented over 3 to 5 months (*14*, *16*). Additionally, short-term repetition of one frequency without behavioral relevance (habituation) results in a dramatic decrease in A1 responses to that frequency [C. D. Condon and N. M. Weinberger, *Behav. Neurosci.* 105, 416 (1991)]. These studies suggest that stimulus presentation without behavioral importance does not result in significant map changes. Although it is unlikely to be a contributing factor, we acknowledge that we did not record from animals that experienced extensive stimulus presentation without any NB stimulation.
20. F. Casamenti, G. Deffenu, A. L. Abbamondi, G. Pepeu, *Brain Res. Bull.* 16, 689 (1986); D. D. Rasmusson, K. Clow, J. C. Szerb, *Brain Res.* 594, 150 (1992); M. E. Jimenez-Capdeville, R. W. Dykes, A. A. Myasnikov, *J. Comp. Neurol.* 381, 53 (1997).
21. B. Hars, C. Maho, J. M. Edeline, E. Hennevin, *Neuroscience* 56, 61 (1993); J. S. Bakin and N. M. Weinberger, *Proc. Natl. Acad. Sci. U.S.A.* 93, 11219 (1996).
22. The cholinergic neurons of the NB were selectively destroyed by infusion of 2.5 μg of 192 immunoglobulin G–saporin immunotoxin into the right lateral ventricle before the surgery to implant the stimulating electrode. The toxin, an antibody to the low-affinity nerve growth factor receptor linked to a ribosome-inactivating toxin, has been shown to specifically destroy most of the cholinergic neurons of the basal forebrain projecting to the cortex, while sparing the parvalbumin-containing neurons as well as cholinergic neurons that project from the NB to the amygdala [S. Heckers *et al.*, *J. Neurosci.* 14, 1271 (1994)]. Electrical stimulation of the NB and tone presentation were identical for lesioned and nonlesioned animals. The percent of the cortex responding to a 19-kHz tone after pairing in lesioned animals was not significantly different from that in naïve controls (two-tailed t test, n = 2).
23. I. Gritti, L. Mainville, M. Mancia, B. E. Jones, *J. Comp. Neurol.* 383, 163 (1997).
24. Complex considerations of the network, cellular, and molecular mechanisms responsible for the plasticity observed in our studies are beyond the scope of this report. See M. E. Hasselmo and J. M. Bower, *Trends Neurosci.* 16, 218 (1993); M. Sarter and J. P. Bruno, *Brain Res. Rev.* 23, 28 (1997); R. W. Dykes, *Can. J. Physiol. Pharmacol.* 75, 535 (1997).
25. Supported by NIH grant NS-10414, Hearing Research Inc., and an NSF predoctoral fellowship. We thank C. Schreiner for technical advice and D. Buonomano, H. Mahncke, D. Blake, C. deCharms, C. Schreiner, and K. Miller for helpful comments on the manuscript.

28 October 1997; accepted 23 January 1998

Activation of the OxyR Transcription Factor by Reversible Disulfide Bond Formation

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The OxyR transcription factor is sensitive to oxidation and activates the expression of antioxidant genes in response to hydrogen peroxide in *Escherichia coli*. Genetic and biochemical studies revealed that OxyR is activated through the formation of a disulfide bond and is deactivated by enzymatic reduction with glutaredoxin 1 (Grx1). The gene encoding Grx1 is regulated by OxyR, thus providing a mechanism for autoregulation. The redox potential of OxyR was determined to be −185 millivolts, ensuring that OxyR is reduced in the absence of stress. These results represent an example of redox signaling through disulfide bond formation and reduction.

Reactive oxygen species can damage DNA, lipid membranes, and proteins and have been implicated in numerous degenerative diseases (*1*). As a defense, prokaryotic and eukaryotic cells have inducible responses that protect against oxidative damage (*2*). These antioxidant defense systems have been best characterized in *Escherichia coli*, in which the OxyR and SoxR transcription factors activate antioxidant genes in response to H_2O_2 and to superoxide-generating compounds, respectively.

The mechanisms of redox-sensing and the systems that control the redox status of the cell are likely to be coupled. Studies of the thiol-disulfide equilibrium of the cytosol of both prokaryotic and eukaryotic cells indicate that the intracellular environment is reducing, such that protein disulfide bonds rarely occur (*3*–*5*). The redox potential of the *E. coli* cytosol has been estimated to be approximately −0.26 to −0.28 V (*4*; *5*). This reducing environment is maintained by the thioredoxin and the glutaredoxin systems (*6*, *7*).

In response to elevated H_2O_2 concentrations, the OxyR transcription factor rapidly induces the expression of *oxyS* (a small, nontranslated regulatory RNA), *katG* (hydrogen peroxidase I), *gorA* (glutathione reductase), and other activities likely to protect the cell against oxidative stress (*2*, *8*). Purified OxyR is directly sensitive to oxidation. Only the xidized form of OxyR can activate transcription in vitro, and footprinting experiments indicate that oxidized and reduced OxyR have different conformations (*9*, *10*). Thus, we examined the chemistry of OxyR oxidation and reduction.

No transition metals were detected by inductively-coupled plasma metal ion analysis of two preparations of OxyR (*11*). We also did not observe any change in OxyR activity after denaturation and renaturation in the presence of the m tal chelator des-

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ferrioxamine (Fig. 1A), indicating that metal ions and other prosthetic groups are unlikely to be the redox-active center of OxyR. Previous mutational studies suggested that at least one and possibly two of the six cysteine residues in OxyR are critical for activity (*12*). We found that the $Cys^{199} \rightarrow Ser^{199}$ (C199S) mutant strain showed no expression of the OxyR-regulated *oxyS* gene, and the $Cys^{208} \rightarrow Ser^{208}$ (C208S) mutant strain only showed slight expression (Fig. 1B). Thus, both Cys^{199} and Cys^{208} are critical to the activation of OxyR. In addition, an alignment of OxyR homologs shows that only two cysteine residues, corresponding to Cys^{199} and Cys^{208} of *E. coli* OxyR, are conserved (*13*).

To examine the oxidation state of the Cys^{199} and Cys^{208} residues in vitro, we generated an OxyR derivative (OxyR4C → A) carrying Ala substitutions of the other four cysteines. This derivative showed activity identical to the wild-type protein in vivo (Fig. 1B) and in vitro (*14*). We examined the OxyR4C → A protein by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry (Fig. 1C). For the reduced protein, two peaks corresponded to fragments containing alkylated Cys^{199} and Cys^{208}. These two peaks completely disappeared for the oxidized protein. Instead, a new peak that corresponded to the sum of the Cys^{199}- and Cys^{208}-containing peptide fragments joined by a disulfide bond, was detected. Quantitative thiol-disulfide titrations also indicated that oxidized OxyR contains one disulfide bond (*15*). We conclude that formation of an intramolecular (*16*) disulfide bond between residues Cys^{199} and Cys^{208} leads to the conformational change that activates the OxyR transcription factor.

Although both Cys^{199} and Cys^{208} were important to OxyR activation, the increased sensitivity of the C199S mutant over the C208S mutant suggested that these two residues are not equivalent. Because the formation of disulfide bonds upon H_2O_2 xidation has been reported to proceed through the initial oxidation of one Cys through a sulfenic acid intermediate (–SOH) (*17, 18*), we propose that the oxidation of Cys^{199} to –SOH is the first step in OxyR activation (*19*).

OxyR activation by H_2O_2 is a transient phenomenon. In a wild-type background, the am unts of *oxyS* reach a maximum ~10 min after H_2O_2 treatment and then decrease to near basal levels within 60 min after the treatment (Fig. 2A). The amounts of the OxyR protein do not change after the H_2O_2 treatment (*9*), suggesting that oxidized OxyR is deactivated by reduction of the Cys^{199}-Cys^{208} disulfide bond. We generated a set of isogenic strains defective in *gorA* (glutathione reductase), *grxA* (Grx1), *gshA* (glutathione synthetase), *trxB* (thioredoxin reductase), and *trxA* (thioredoxin)—the components of the two main disulfide reduction systems in the cell. We then examined the activity of OxyR. Compared to the wild-type strain, *oxyS* RNA levels were elevated 30 min after H_2O_2 treatment in the *gorA*$^-$, and particularly the *grxA*$^-$ and *gshA*$^-$ mutants. By contrast, the *trxA*$^-$ mutant showed a profile identical to the wild-type strain (*14*), and the *trxB*$^-$ mutant exhibited a more rapid decrease in *oxyS* expression, possibly because of increased Grx1 levels in this strain. Because Grx1 is known to catalyze protein disulfide bond reduction by reduced glutathione (GSH), Grx1 may catalyze OxyR deactivation at the expense of GSH. To test this hypothesis, we incubated oxidized OxyR with purified GSH and Grx1. OxyR activity was completely eliminated within 30 min (Fig. 2B, lane 4). OxyR deactivated in this manner could readily be reactivated by H_2O_2 upon removal of GSH (lane 5). These genetic and biochemical results indicate that OxyR is deactivated through enzymatic disulfide bond reduction by Grx1.

OxyR was initially identified as a sensor for H_2O_2 levels. However, the OxyR-regulated *katG* gene has also been reported to be induced by diamide and *S*-nitrosothiols (*20*). To test the OxyR sensing specificity, we treated cells with H_2O_2, diamide, *S*-nitroso-

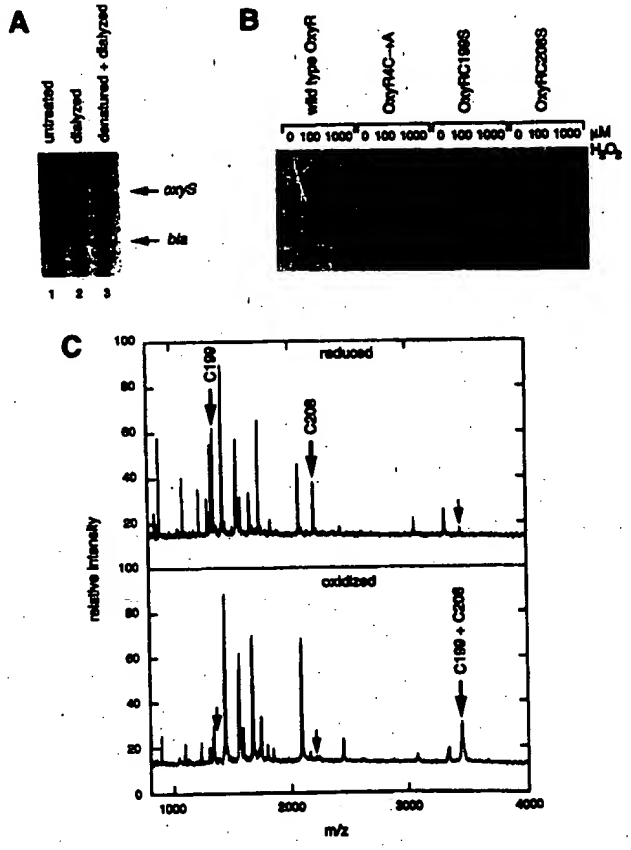

Fig. 1. Direct activation of OxyR. (**A**) The buffer in a sample of OxyR (1 ml of ~0.5 mg/ml) was exchanged by three additions (1 ml) of 6 M guanidine hydrochloride in 0.1 M potassium phosphate (pH 7.0) and concentrated to 50 μl in a Centricon unit (10 kD cutoff, Amicon). Circular dichroism measurements confirmed that the OxyR protein was denatured by the guanidine hydrochloride. An aliquot of denatured OxyR was then renatured by a 6-hour dialysis against three 100-ml volumes of protein purification buffer containing the metal chelator desferrioxamine (0.1 mM, Sigma). Subsequently, equal amounts of an untreated sample (lane 1), a sample dialyzed against 0.1 M potassium phosphate, pH 7.0 (lane 2), and the denatured and dialyzed sample (lane 3) were analyzed by in vitro transcription assays using purified RNA polymerase (U.S. Biochemical) and pAQ17 as a template. OxyR was purified as described (*12*), with the exception that dithiothreitol (DTT) was eliminated from the purification buffer. All transcription reactions (*12*) were carried out with ≤1 μM OxyR to ensure a linear response. (**B**) Strains expressing wild-type OxyR, OxyR4C→A, OxyRC199S, and OxyRC208S (on pUC plasmids) were grown to an optical density at 600 nm (OD_{600}) = 0.2 in LB medium and then treated with 0, 100, or 1000 μM H_2O_2. Total RNA was isolated from samples taken at 10 min, and the amounts of *oxyS* RNA were analyzed by primer extension (5′-CGTTTTCAAGGCCC) (*8*). (**C**) MALDI-TOF spectra for reduced (top) and oxidized (bottom) OxyR4C→A after alkylation and trypsin digestion were taken with a LaserTec Bench-Top (VESTEC) mass spectrometer. All the predicted tryptic fragments could be identified in the 800- to 4000-dalton region, and the mass of each observed fragment differed from its theoretical value by <1 dalton. To prepare the samples, we first alkylated reduced and oxidized OxyR4C→A [100 μl of 0.5 mg/ml, purified as in (A) after extraction from inclusion bodies] by a 10-min incubation with iodoacetamide (1 μl of 1 M in H_2O, Sigma). Trypsin (1 μl of 1 mg/ml, Promega) was added to the alkylated protein (25 μl of ~0.5 mg/ml), and the mixture was incubated overnight at 37°C. Subsequently, 1 μl of the digestion product was added to 3 μl of saturated 2,5-dihydroxybenzoic acid (Aldrich) in a 2:1 0.1% trifluoroacetic acid/acetonitrile solvent mixture, and 1 μl of this mixture was loaded onto the sample pin of the spectrometer. The generation of reduced OxyR4C→A protein and all subsequent manipulations were carried out in an anaerobic chamber (Coy Laboratory) filled with 5% H_2 and 95% N_2.

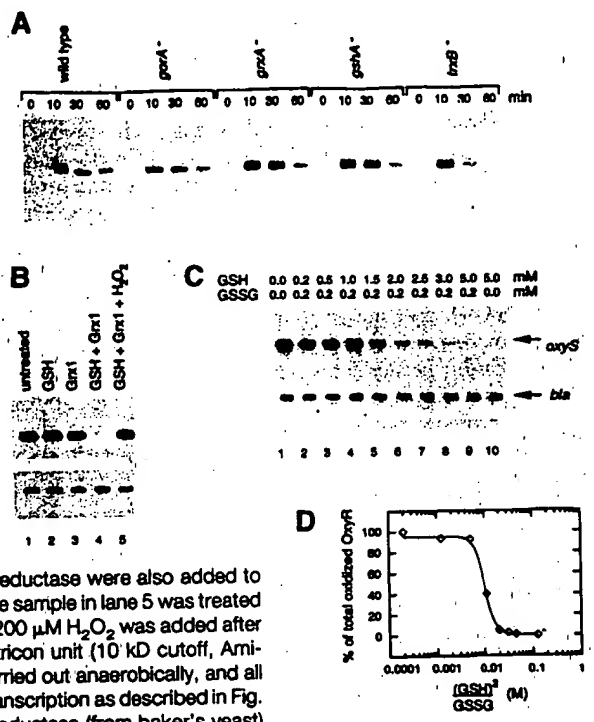

Fig. 2. OxyR deactivation by Grx1. (**A**) The *gorA*⁻, *grxA*⁻, *gshA*⁻, *trxB*⁻, and *trxA*⁻ mutant alleles (*27*) were moved into MC4100 by P1 transduction (generating GSO48-GSO52). The strains were grown to OD_{600} = 0.2 in minimum M63 medium supplemented with 0.2% glucose and 0.002% vitamin B1 and then treated with 200 μM H_2O_2. Total RNA isolated from samples taken at 0, 10, 30, and 60 min was analyzed by primer extension as in Fig. 1. The data shown is representative of the average (2.3, 1.8, 0.5, 0.5, 2.4, and 2.0% decay/min for wild type, *gorA*⁻, *grxA*⁻, *gshA*⁻, *trxB*⁻, and *trxA*⁻, respectively) of 10 experiments. (**B**) Samples of purified OxyR (0.8 μM) were incubated with 5 mM GSH (lane 2), 10 μM Grx1 (lane 3), 5 mM GSH and 10 μM Grx1 (lane 4) for 30 min. NADPH (0.5 mM) and 10 μg/ml glutathione reductase were also added to the samples in lanes 2 through 5. The sample in lane 5 was treated as described for lane 4 except that 200 μM H_2O_2 was added after the GSH was removed with a Centricon unit (10 kD cutoff, Amicon). The entire experiment was carried out anaerobically, and all samples were analyzed by in vitro transcription as described in Fig. 1. NADPH, GSH, and glutathione reductase (from baker's yeast) were purchased from Sigma, and Grx1 was kindly provided by J. Bushweller and A. Holmgren. The results shown are representative of four independent experiments. (**C**) The indicated amounts of GSH and GSSG were incubated with 0.8 μM OxyR and 10 μM Grx1 at pH 7 and 27°C for at least 72 hours. The samples were then added to RNA polymerase and assayed by in vitro transcription. All steps were carried out anaerobically. (**D**) The intensities of the *oxyS* and *bla* bands in (C) were measured by a PhosporImager (Molecular Dynamics) and then converted to OxyR (oxidized) concentration, using a calibration curve obtained from a control experiment in which a total of 0.8 μM OxyR composed of defined amounts of oxidized and reduced OxyR was assayed by in vitro transcription under the same conditions used for the titration. The diamonds correspond to experimental data, and the solid line is the theoretical fit {% oxidized OxyR = $K_{eq}/(K_{eq} + [GSH]^4/[GSSG]^4)$} based on Eq. 1. The redox potential of −185 ± 5 mV is derived from three independent experiments.

cysteine (SNO-Cys), nitrite, hydrazine and its derivatives, hypochlorous acid, and oxidized lipoic acid (*14*). Only H_2O_2 and diamide activated OxyR in the wild-type strain, and diamide activation was only observed at concentrations greater than 100 μM (Fig. 3A). SNO-Cys did activate OxyR in a *gshA*⁻ strain, but activati n by SNO-Cys was always lower than the activation by H_2O_2 (Fig. 3A). In vitro, diamide, SNO-Cys, and oxidized glutathione (GSSG) all partially activated OxyR but to significantly lower amounts than did H_2O_2 (Fig. 3B). Thus, although diamide and SNO-Cys might react with the two critical cysteines in OxyR, the transcription factor seems to have evolved to specifically sense peroxides.

The reversible reaction between OxyR and GSH/GSSG (Fig. 3B) allowed us to measure the redox potential of OxyR. We incubated OxyR with defined concentrations of GSH/GSSG and then measured the relative amounts of oxidized (activated) OxyR by in vitro transcription assays (Fig. 2C). When the GSH:GSSG ratio in the buffer exceeded 5:1 (between lanes 4 and 6), there was a sudden and substantial drop in transcription activity. This titration data (Fig. 2D) was best fit by assuming a concerted four-monomer oxidation and reduction (Eq. 1), which is consistent with the observation that OxyR is a tetramer in solution (*21*).

$$\text{OxyR(ox)} + 8\,\text{GSH} \xleftrightarrow{\text{Grx1}} \text{OxyR(red)} + 4\,\text{GSSG} \quad (1)$$

The extracted equilibrium constant for Eq. 1 was used to calculate the redox potential of OxyR (*22*). The derived value of −185 ± 5 mV is about 90 mV higher than the estimated redox potential of the *E. coli* cytosol (−280 mV) (*4*, *5*), providing a ther-

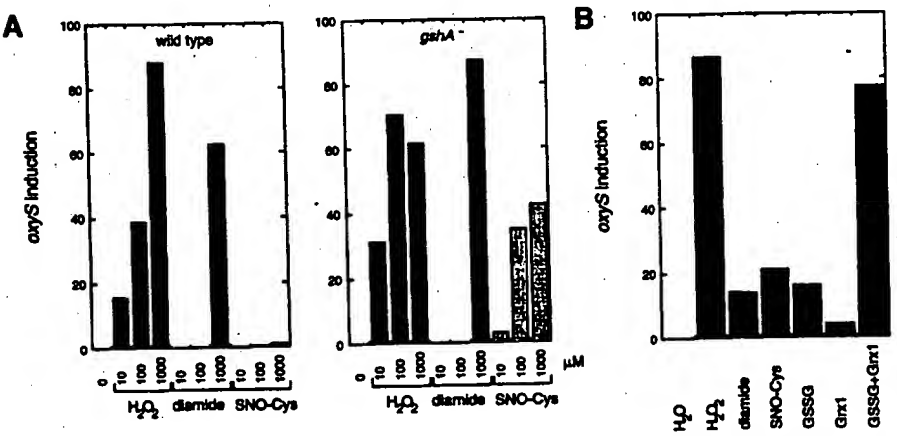

Fig. 3. Specificity of OxyR oxidation. (**A**) The wild-type (MC4100) and *gshA*⁻ (GSO49) strains were grown in minimal medium as in Fig. 2 and then treated with 10, 100, or 1000 μM H_2O_2, diamide (Sigma), and SNO-Cys [synthesized according to (*28*)]. Total RNA was isolated from cells collected after 10 min, and primer extension assays were carried out as in Fig. 1. The *oxyS* levels were quantified on a PhosphorImager and plotted. (**B**) Purified OxyR was reduced by overnight treatment with 0.1 mM DTT, which was then removed by dialysis (Pierce dialysis cassette). The samples (0.8 μM) were then treated with 200 μM H_2O_2, 200 μM diamide, 200 μM SNO-Cys, 200 μM GSSG (Sigma), 10 μM Grx1, and 200 μM GSSG plus 10 μM Grx1 for 5 min and assayed by in vitro transcription. The entire experiment was carried out anaerobically. The in vivo and in vitro assays were both repeated at least twice; representative experiments are shown.

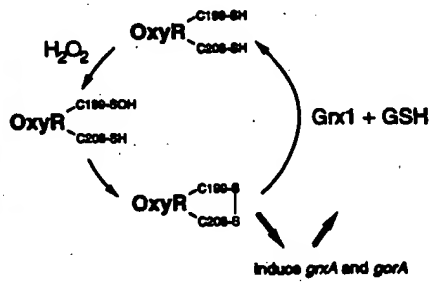

Fig. 4. Model for OxyR activation and deactivation. Upon exposure to H_2O_2, the Cys^{199} residue of OxyR is first oxidized to a sulfenic acid. This reactive intermediate subsequently reacts with Cys^{208} to form a stable disulfide bond locking OxyR in an activated form. Oxidized OxyR is re-reduced by disulfide bond reduction by the glutaredoxin system. Because OxyR activates the transcription of *grxA* (Grx1) and *gorA* (glutathione reductase), the entire response is autoregulated.

modynamic basis for the observation that OxyR is predominantly reduced (deactivated) under normal conditions. The redox potential of OxyR is also higher than the potential of all the known disulfide reductases in *E. coli* (7). Thus, thioredoxin should also be capable of reducing OxyR, and indeed, we found that the purified enzyme deactivates OxyR in vitro (23). Because the OxyR protein is eventually reduced in *gorA*$^-$, *grxA*$^-$, and *gshA*$^-$ mutant strains, it is also likely that the other disulfide bond reduction systems contribute to the deactivation of OxyR in vivo.

Interestingly, an examination of the promoter region of the *grxA* gene revealed a sequence that showed an 85% match to an OxyR DNA-binding consensus sequence (10). We examined the levels of the *grxA* message by primer extension and found that, as previously observed for *gorA*, the expression of *grxA* is induced by H_2O_2 in an OxyR-dependent manner (24). Deoxyribonuclease I (DNase I) footprinting experiments also showed that the OxyR footprint precisely overlaps the predicted OxyR binding site (24). These results indicate that the OxyR response is autoregulated; OxyR activation by H_2O_2 leads to the induction of activities that trigger the OxyR deactivation pathway.

We have provided evidence that the molecular event of redox signaling by OxyR is disulfide bond formation and reduction (Fig. 4). Two features of OxyR are likely to contribute to its sensitivity to H_2O_2. First, the oxidation and reduction of OxyR tetramers appears to be cooperative. Second, we suggest that the Cys^{199} residue is poised to react with H_2O_2. A comparison of OxyR homologs reveals that two basic residues flanking Cys^{199} are absolutely conserved (13). These residues could enhance the reactivity of Cys^{199} toward peroxides by stabilizing the thiolate form of this cysteine (Cys^{199}-S$^-$) or by protonating the leaving group (–OH) of H_2O_2, or both.

OxyR induction of Grx1 and glutathione reductase ensures that the transcription factor is only activated for a defined period of time and may also be a mechanism for cells to maintain redox homeostasis. A drop in the GSH:GSSG ratio could lead to OxyR activation resulting in the induction of the enzymes that restore the redox balance. Because GSH:GSSG ratios vary significantly from one intracellular compartment to another in eukaryotic cells, a variety of cellular processes, including signal transduction and transport, may be modulated by reversible disulfide bond formation.

The redox potential of –185 mV determined for OxyR is substantially higher than the redox potential of –285 mV reported for the SoxR transcription factor (25). We propose that whereas the activity of OxyR is responsive to the thiol-disulfide redox status of the cell, the activity of SoxR is responsive to reduced and oxidized nicotinamide adenine dinucleotide phosphate (NADPH/NADP$^+$, respectively) levels in the cell. In general, the difference in the redox potential of the two major intracellular redox buffers (GSH/GSSG and NADPH/NADP$^+$) (4) should allow for the regulation of proteins with chemically diverse redox centers.

REFERENCES AND NOTES

1. B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine* (Clarendon Press, Oxford, 1989).
2. D. J. Jamieson and G. Storz, in *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*, J. G. Scandalios, Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997), pp. 91–115; B. González-Flecha and B. Demple, in *Reactive Oxygen Species in Biological Systems: An Interdisciplinary Approach*, D. L. Gilbert and C. A. Colton, Eds. (Plenum, New York, in press).
3. G. E. Schultz and R. H. Schirmer, *Principles of Protein Structure* (Springer-Verlag, New York, 1979); J. M. Thornton, *J. Mol. Biol.* 151, 261 (1981); C. Branden and J. Tooze, *Introduction to Protein Structure* (Garland, New York, 1991).
4. H. F. Gilbert, *Adv. Enzymol. Relat. Areas Mol. Biol.* 63, 69 (1990).
5. C. Hwang, A. J. Sinskey, H. F. Lodish, *Science* 257, 1496 (1992).
6. A. I. Derman, W. A. Prinz, D. Belin, J. Beckwith, *ibid.* 262, 1744 (1993); W. A. Prinz, F. Åslund, A. Holmgren, J. Beckwith, *J. Biol. Chem.* 272, 15661 (1997).
7. F. Åslund, K. D. Berndt, A. Holmgren, *J. Biol. Chem.* 272, 30780 (1997).
8. S. Altuvia, D. Weinstein-Fischer, A. Zhang, L. Postow, G. Storz, *Cell* 90, 43 (1997).
9. G. Storz, L. A. Tartaglia, B. N. Ames, *Science* 248, 189 (1990).
10. M. B. Toledano *et al.*, *Cell* 78, 897 (1994).
11. Two samples of OxyR (15 μM) were analyzed by the Chemical Analysis Laboratory at the University of Georgia. No transition metals were detected at a sensitivity of ≤5 pM.
12. I. Kullik, M. B. Toledano, L. A. Tartaglia, G. Storz, *J. Bacteriol.* 177, 1275 (1995).
13. *Escherichia coli* (J04553, residues 191-208), LLMLEDGHCLRDQAMGFC; *Erwinia carotovora* (U74302), LLMLEDGHCLRDQAMGFC; *Haemophilus influenzae* (U49355), MLMLDDGHCLRNQALDYC; *Xanthomonas campestris* (U94336), LLLLEDGHCLRDQALDVC; *Mycobacterium leprae* (L01095), LLLLDEGHCLRDQTLDIC; *Acinetobacter calcoaceticus* (X88895), LMLLEEGHCLRDHALSAC (26).
14. M. Zheng, F. Åslund, G. Storz, data not shown.
15. We carried out 5,5′-dithiobis-(2-nitrobenzoic acid) and 2-nitro-5-thiosulfobenzoate titrations under denaturing conditions as described [P. W. Riddles, R. L. Blakeley, B. Zerner, *Anal. Biochem.* 94, 75 (1979); T. W. Thannhauser, Y. Konishi, H. A. Scheraga, *ibid.* 138, 181 (1984)]. For reduced OxyR4C→A, 1.8 equivalents of –SH and 0.1 equivalents of S-S were detected, whereas 0.2 equivalents of –SH and 0.9 equivalents of S-S were detected for the oxidized protein.
16. Mass spectrometry (MALDI-TOF) of oxidized, denatured OxyR revealed a major peak at 34.3 ± 0.1 kD and a minor peak at 17.1 ± 0.1 kD, corresponding to the singly- and doubly-charged OxyR monomer (theoretical mass of 34.28 kD), respectively.
17. J. L. Kice, *Adv. Phys. Org. Chem.* 17, 65 (1980); A. Claiborne, H. Miller, D. Parsonage, R. P. Ross, *FASEB J.* 7, 1483 (1993).
18. F. A. Davis and R. L. Billmers, *J. Am. Chem. Soc.* 103, 7016 (1981); H. R. Ellis and L. B. Poole, *Biochemistry* 36, 13349 (1997).
19. When we examined the trypsin-digested C208S mutant protein by mass spectrometry, we observed a 2614 dalton peak for the oxidized but not for the reduced C208S protein. The mass of this peak corresponds to that of two Cys^{199}-containing fragments linked via a thiosulfinate functional group, which would be formed by a sulfenic acid condensation reaction [2 Cys^{199}-SOH → Cys^{199}-S(O)S-Cys^{199} + H_2O] [(17); E. Block and J. O'Connor, *J. Am. Chem. Soc.* 96, 3929 (1974); F. A. Davis, L. A. Jenkins, R. L. Billmers, *J. Org. Chem.* 51, 1033 (1986)].
20. C. T. Privalle and I. Fridovich, *J. Biol. Chem.* 265, 21966 (1990); A. Hausladen, C. T. Privalle, T. Keng, J. DeAngelo, J. S. Stamler, *Cell* 86, 719 (1996).
21. I. Kullik, J. Stevens, M. B. Toledano, G. Storz, *J. Bacteriol.* 177, 1285 (1995).
22. The equilibrium constant K_{eq} extracted from the redox titration data shown in Fig. 2C was used to calculate the redox potential of OxyR according to the Nernst equation:

$$E = E^0 + 2.303(RT/nF) \cdot \log K_{eq}$$

where E is the redox potential of OxyR in reference to the normal hydrogen electrode, E^0 is the standard potential of GSH [–240 mV (7)], R is the gas constant, T is the temperature, n is the number of electrons transferred (eight electrons in the case of OxyR), and F is the Faraday constant.
23. OxyR reduction by thioredoxin reductase and thioredoxin was carried out essentially the same as the reduction described in Fig. 2B, except that 10 μg/ml *E. coli* thioredoxin reductase (trxc) and 10 μM *E. coli* thioredoxin (trxc) were used in place of glutathione reductase, Grx1, and GSH.
24. The OxyR-regulated transcription start was mapped to an A residue 23 nucleotides upstream of the AUG. The extent of the OxyR DNase I footprint on both strands is underlined, and the matches to the OxyR binding site consensus (10) are capitalized on the following sequences. Top strand: 5′- gtgtttaacagtTATAGcTttAgCaATaatcgcAcAGgTtaaAcCTActttcagcgaa; bottom strand: 3′- cacaaattctcaaTATCcAaaaTcGtTAattacgtTgTCcAatTgGATgaaagtcgctt. The same transcription start and OxyR binding site were recently identified [K. Tao, *J. Bacteriol.* 179, 5967 (1997)].
25. H. Ding, E. Hidalgo, B. Demple, *J. Biol. Chem.* 271, 33173 (1996); P. Gaudu and B. Weiss, *Proc. Natl. Acad. Sci. U.S.A.* 93, 10094 (1996); E. Hidalgo, H. Ding, B. Demple, *Cell* 88, 121 (1997).
26. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
27. N. K. Davis, S. Greer, M. C. Jones-Mortimer, R. N. Perham, *J. Gen. Microbiol.* 128, 1631 (1982); J. T. Greenberg and B. Demple, *J. Bacteriol.* 168, 1026 (1986); M. Russel and A. Holmgren, *Proc. Natl. Acad. Sci. U.S.A.* 85, 990 (1988); M. Russel and P. Model, in *Thioredoxin and Glutaredoxin Systems: Structure and Function*, A. Holmgren, C. I. Brändén, H. Jörnvall, B.-M. Sjöberg, Eds. (Raven Press, New York, 1986), pp. 331–337.
28. J. S. Stamler and J. Loscalzo, *Anal. Chem.* 64, 779 (1992).

29. We thank C. Wu and C. Klee for the use of the MALDI mass spectrophotometer and Pharmacia Smart system, C. Vinson for use of the spectropolarimeter, L. Poole and J. Beckwith (grant GM-41883) for experiments conducted by M.Z. and F.A. in their laboratories, and J. Bushweller, B. Demple, A. Eisenstark, A. Holmgren, and M. Russel for *E. coli* strains, plasmids, and purified Grx1. We also appreciate the advice of J. Beckwith, L. Poole, and W. Prinz, and the editorial comments of J. Beckwith, C. Dismukes, R. Klausner, L. Poole, C. Wu, Y.-L. Wu, and M. Zhong. Supported by the intramural program of the National Institute of Child Health and Human Development and grants from the American Cancer Society (M.Z.), the Karolinska Institute (F.A.), and the Wennergren Foundation (F.A.).

3 September 1997; accepted 6 January 1998

JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6181–6186
0021-9193/97/$04.00+0

Vol. 179, No. 19

Transcriptional Regulation of the *Escherichia coli oxyR* Gene as a Function of Cell Growth

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Received 9 April 1997/Accepted 30 July 1997

The *oxyR* regulon plays a central role in the defense of *Escherichia coli* against the endogenous oxidative damage associated with active aerobic growth. Here we have studied the transcriptional regulation of *oxyR* in *E. coli* growing aerobically in rich medium. Expression of a single-copy *oxyR'::lacZ* reporter construct varied sixfold along the growth curve, with the highest value at 4 to 6 h of growth (~14 × 10^8 cells · ml^{-1}). Direct measurements of *oxyR* mRNA by primer extension showed the same biphasic expression but with a peak somewhat earlier in cell growth (2 to 3 h; ~3.5 × 10^8 cells · ml^{-1}). The results of immunoblotting experiments demonstrated that the level of OxyR protein exhibits the same biphasic expression. Mutant strains lacking adenylate cyclase (*cya*) or Crp protein (*crp*) failed to increase *oxyR* expression during exponential growth. On the other hand, an *rpoS* mutation allowed *oxyR* expression to continue increasing as the cells entered stationary phase. Consistent with a biological role for increased levels of OxyR during exponential growth, the *crp cya* strain had lower activities of catalase hydroperoxidase I and glutathione reductase and an increased sensitivity to exogenously added hydrogen peroxide. These results suggest that the Crp-dependent upregulation of *oxyR* in exponential phase is a component of a multistep strategy to counteract endogenous oxidative stress in actively growing *E. coli* cells.

The adaptive responses to oxidative stress in *Escherichia coli* include two regulons controlled by the *oxyR* and *soxRS* genes, which orchestrate defense gene induction triggered by hydrogen peroxide or superoxide-generating systems, respectively (15). Exposing wild-type *E. coli* to micromolar levels of H_2O_2 induces a protective response that confers resistance to subsequent exposure to millimolar H_2O_2 concentrations (6, 8). About 8 of the 30 to 40 proteins induced after treatment with H_2O_2 are products of the *oxyR* regulon genes (6, 13, 25).

To date, the possible transcriptional regulation of the *oxyR* gene has not been extensively studied, except to note that the level of *oxyR* mRNA was not increased after H_2O_2 activation of the *oxyR* regulon (7). Although overproduction of OxyR from multicopy plasmids was not followed by superinduction of the OxyR-dependent enzymes upon H_2O_2 treatment (30), several lines of evidence suggest that regulation of *oxyR* expression has an important biological role. For example, the glucose repression described for catalase (13, 25) may not arise from direct regulation of *katG* by Crp, since no functional Crp-binding site has been found in the *katG* promoter. Also, the OxyR-mediated response to equivalent H_2O_2/catalase ratios varies along the growth curve, being maximal during exponential phase and almost negligible during early stationary phase (11). Finally, the close correlation between the rates of production of O_2^- and H_2O_2 and the respiratory activity of bacterial cells (11), together with the role for OxyR in the homeostatic regulation of the intracellular concentration of H_2O_2 (12), points to a regulation of OxyR activity by oxygen tension or respiration.

In this study, we monitored the variation of *oxyR* expression in aerobically growing bacteria and evaluated possible regulators of the *oxyR* gene. We also measured the level of OxyR-dependent activities and the sensitivity to H_2O_2 in strains with decreased expression of *oxyR*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists the *E. coli* strains and plasmids used in this study. The bacteriophage λRS45 (*bla'-'lacYA*$^+$) (29) was used to insert the *oxyR'::lacZ* fusion into the chromosomal DNA by recombination in strain MC4100 carrying plasmid pAQ23, using methods previously described (29). Plasmid pAQ23 contains the 5' 15% of *oxyR* cloned into pRS415 (7). The resulting lysogens, BGF930, BGF932, and BGF940, were identified by their Lac$^+$ Amp^s phenotype. Attempts to introduce the single-copy fusion into strains CA8000, CA8306, and CA8445 were unsuccessful. Strain BGF612 was constructed by cotransduction (24) of Δ*cya* (50% linked to Km^r) into strain BGF930. Strains BGF950, BGF960, and BGF970 were constructed by cotransduction into strain MC4100 of Tn*10* markers linked to the *fnr-250*, *arcB1*, and *arcA2* alleles in strains RK5288, ECL594, and ECL906, respectively. Strain BGF1030 was constructed by transduction of *katF1::kan* from strain ZK1001 into BGF930.

Cells were inoculated into LB broth (24) containing the appropriate antibiotics and incubated overnight at 37°C with gentle shaking (100 rpm). For experiments, the cultures grown overnight were diluted 100-fold into fresh LB broth and incubated at 37°C for the times indicated in the figures. Antibiotics were used at the following concentrations (in micrograms per milliliter): ampicillin, 100; tetracycline, 12.5; streptomycin, 50; and kanamycin, 50.

Anaerobic cell growth was performed in 2-ml vials fitted with butyl rubber stoppers as previously described by Jones and Gunsalus (20).

β-Galactosidase. β-Galactosidase activity in sodium dodecyl sulfate (SDS)-CH_3Cl-treated cells was determined by the method of Miller (24) and normalized to the cell density. Absorbance and optical density measurements were carried out in a Perkin-Elmer model Lambda 3A spectrophotometer (Perkin-Elmer, Oak Brook, Ill.).

RNA isolation and primer extension. Samples containing ~10^{10} cells (5 to 20 ml) were taken at the indicated times of growth and placed on ice, and total RNA was extracted by a modified version of the hot-phenol extraction method of Emory and Belasco (9). Briefly, the chilled samples were centrifuged and the cells were resuspended in 125 μl of ice-cold 0.3 M sucrose–0.01 M sodium acetate (pH 4.5). After addition of 125 μl of 2% SDS–0.01 M sodium acetate (pH 4.5), the cell suspension was heated for 3 min at 70°C and extracted three times for 3 min each at 70°C with 250 μl of hot phenol previously equilibrated with unbuffered water. The RNA was ethanol precipitated and stored at −80°C in 20 mM sodium phosphate (pH 6.5)–1 mM EDTA. The RNA concentration in a sample was determined spectrophotometrically (28). A 20-base oligonucleotide (5'-GGTACTCAAGATCACGAATA-3') was 5' end labeled with polynucleotide kinase and [γ-^{32}P]ATP (~8,000 Ci · $mmol^{-1}$). RNA samples (100 μg) were mixed with the labeled primer (15,000 to 30,000 cpm) in water, and the samples

TABLE 1. Bacterial strains and plasmids used in this study

E. coli strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
BGF930	As RK4936, but λ[Φ(*oxyR'::lacZ*)]	This work
BGF931	As RK4936, but λ[Φ(*katG'::lacZ*)]	11
BGF932	As TA4112, but λ[Φ(*oxyR'::lacZ*)]	This work
BGF933	As TA4112, but λ[Φ(*katG'::lacZ*)]	11
BGF940	As MC4100, but λ[Φ(*oxyR'::lacZ*)]	This work
BGF950	As MC4100, but λ[Φ(*oxyR'::lacZ*)] *fnr-250 zcj*::Tn*10*	This work
BGF960	As MC4100, but λ[Φ(*oxyR'::lacZ*)] *arcB1 zgf*::Tn*10*	This work
BGF612	As BGF930, but Δ*cya* Km^r	This work
BGF970	As MC4100, but λ[Φ(*oxyR'::lacZ*)] *arcA2 zjj*::Tn*10*	This work
BGF1030	As BGF930, but *katF1*::*kan*	This work
CA8000	Hfr H B_1^-	4
CA8306	Hfr H B_1^- Δ*cya*	27
CA8445	Hfr H B_1^- Δ*cya* Δ*crp*	27
ECL594	As ECL547, but *arcB1 zgf*::Tn*10*	16
ECL906	As ECL525, but *arcA2 zjj*::Tn*10*	17
MC4100	Δ(*lac*)*U169 rpsL*	Laboratory stock
M7141	Δ*cya* 50% linked to Km^r	A. Hochschild
RK4936	*araD139* (*argF-lac*)*205 flbB5301 non-9 gyrA219 relA1 rpsL150 metE70 btuB*::Tn*10*	Laboratory stock
RL5288	As MC4100, but *gyrA non* λp1(*209*) Δ(Mu) Φ(*nar-lac*)*218 fnr-250 zcj-637*::Tn*10*	20
RR1	*pro leu rpsL hsdM hsdR endI lacY*	3
TA4112	As RK4936, but Δ(*oxyR-btuB*)*3*	6
ZK1001	As W3110, but Δ*lacU169 tnaZ katF1*::*kan cysC95*::Tn*10*	R. Kolter
Plasmids		
pAT153	Vector Ap^r Tc^r	33
pUC18	Vector Ap^r	35
pUC19	Vector Ap^r	Laboratory stock
pRS415	Vector Ap^r	Laboratory stock
pAQ17	pUC19 containing *oxyR*	7
pAQ23	pRS415 containing *oxyR'::lacZ*	7
pAQ24	pRS415 containing *katG'::lacZ*	31
pBT22	pAT153 containing *katG*	32
pDEB2	pUC19 containing *rpoS*	2

were heated at 58°C for 5 min and then cooled on ice for 10 min. After this annealing procedure, extension products were generated with the avian myeloblastosis virus reverse transcriptase (Promega, Middleton, Wis.) following the manufacturer's procedure. The products were electrophoresed in denaturing gels containing 8% acrylamide (28).

Immunoblot assay of OxyR. Pellets from 5- to 20-ml cultures (~10^{10} cells) were sonicated in 250 μl of 10 mM Tris buffer (pH 8) containing 20% glycerol, centrifuged at 6,000 × *g* for 15 min, and concentrated 10-fold by evaporation. Samples (~200 μg of total protein) were electrophoresed in an SDS-12% polyacrylamide gel and electroblotted to a nitrocellulose filter. OxyR was detected by using antibodies against an OxyR–β-galactosidase fusion protein (30) and visualized with the enhanced chemiluminescence system (Amersham, Arlington Heights, Ill.).

H_2O_2 concentrations. The intracellular concentration of H_2O_2 was assessed by peroxidase-mediated scopoletin oxidation as previously described (12).

RESULTS

Growth-phase-dependent variation in *oxyR* expression. We monitored *oxyR* expression during growth by introducing a single-copy *oxyR'::lacZ* operon fusion into wild-type (BGF930) and Δ*oxyR* (BGF932) strains. β-Galactosidase activity showed a biphasic profile in both strains, with minimal values at ~2 h of outgrowth, and maxima at ~4 h (Fig. 1A). After reaching a peak at mid-exponential phase, *oxyR* expression declined, yielding plateau values of ~200 and ~400 U of β-galactosidase for BGF930 and BGF932, respectively (Fig. 1A).

The *oxyR'::lacZ*-directed β-galactosidase activity in the Δ*oxyR* strain (BGF932) was always somewhat higher than that of the wild-type strain (BGF930). This result indicates that the autorepression of *oxyR*, reported by Christman et al. (7) for exponential-phase cultures, does not alter the oscillating pattern of *oxyR* expression during aerobic growth.

We confirmed the results obtained with the reporter fusion by measuring the levels of *oxyR*$^+$ message directly. The steady-state level of *oxyR* mRNA, assessed by primer extension, was 3.5-fold higher during exponential growth (2 to 4 h) than the initial level (Fig. 1B). A similar result was obtained by using Northern blotting to detect *oxyR* mRNA (data not shown). The biphasic profile was similar in shape to that for the *oxyR'::lacZ* fusion. However, the induction and maximum for the mRNA level preceded by ~2 h that seen for β-galactosidase expression with the fusion gene (Fig. 1A). This difference is likely due to a delay for protein synthesis and effects of the stability of β-galactosidase.

We checked whether the level of OxyR protein in the bacteria reflected the observed variations in mRNA levels during growth. Polyclonal antibodies against OxyR (30) were employed in immunoblotting experiments with protein samples normalized to 10^9 cells. A typical experiment showed that very little OxyR was present at 1 h of outgrowth and that the protein accumulated steadily through exponential phase and into early stationary phase (Fig. 1C). The OxyR protein level then decreased during stationary phase, with a slight but reproducible increase at 16 h (Fig. 1C). Overall, the pattern of OxyR protein expression paralleled the transcriptional behavior of the *oxyR* gene reasonably well.

Transcriptional regulation of *oxyR*. Since OxyR mediates a response to oxidative stress in *E. coli*, we first evaluated the possibility of oxygen dependence of its growth-phase-dependent regulation. The change in *oxyR* expression during aerobic growth was completely abolished in the absence of oxygen (Fig. 2). Neither addition of exogenous H_2O_2 (7, 30) nor treatment with superoxide-generating systems (unpublished results) affected *oxyR'::lacZ* expression. Thus, neither OxyR nor SoxRS activates the *oxyR* gene. These results and the close relation between the metabolic production of oxygen radicals and the respiratory activity of the bacterial cell (11) prompted us to evaluate the roles of the major global regulators of aerobic gene expression in *E. coli* in the control of *oxyR*. The systems examined included ArcAB, Fnr, Crp (Cap, cyclic AMP [cAMP] receptor protein) and changes in RNA polymerase σ subunits.

Anaerobic cultures of strains BGF960 (*arcB1*) and BGF970 (*arcA2*) displayed higher β-galactosidase activity than the parental strain BGF940 (Fig. 3). However, this effect may be indirect, since neither the phosphorylated or unphosphorylated form of ArcA protein bound to the *oxyR* promoter region in vitro (23). An Fnr$^-$ phenotype (strain BGF950) did not significantly alter *oxyR'::lacZ* expression in anaerobic cells (Fig. 3). Similar relative profiles for the various strains were observed for aerobic cultures of the strains (Fig. 3).

The presence of near-consensus half-sites (5) for Crp (TGTGA-N_6-TCAGT) in the −62 to −46 region of the *oxyR* promoter (7), together with the glucose effect reported for *katG* expression (14), prompted us to evaluate the possible effects of cAMP and Crp on *oxyR* expression. Addition of glucose (to 0.2% in the growth medium) decreased *oxyR'::lacZ* expression in BGF930 (*oxyR*$^+$) to ~30 U of β-galactosidase at all stages of aerobic growth. The steady-state H_2O_2 concentration was reduced in cells grown in LB medium with glucose to

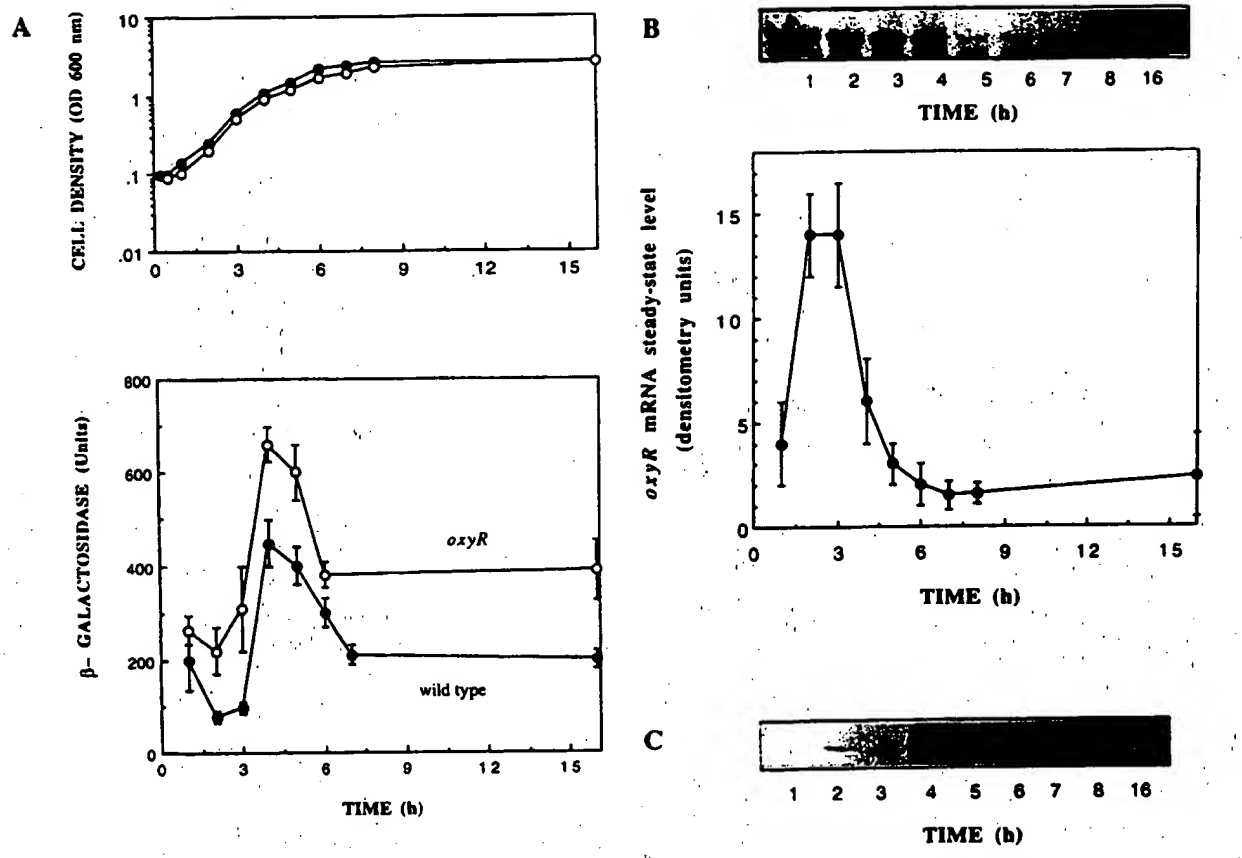

FIG. 1. Variation in *oxyR* expression during aerobic growth. (A) Expression of a single-copy *oxyR'::lacZ* operon fusion. Cultures of BGF930 (wild type; filled circles) and BGF932 (Δ*oxyR*; open circles) grown overnight were diluted 100-fold in fresh LB medium and incubated at 37°C with gentle shaking. At the indicated times, samples were taken to assay β-galactosidase activity (bottom). The top panel displays typical growth curves. OD 600 nm, optical density at 600 nm. (B) *oxyR* mRNA levels determined by primer extension. Strain BGF930 (*oxyR*$^+$) was grown and sampled as described above, except that the samples were processed for RNA measurements (see Materials and Methods). A representative blot (top) and densitometric quantification of three independent experiments (bottom) are shown. rRNA bands stained with ethidium bromide were used as a reference to confirm equal loading. (C) OxyR protein levels. Strain RK4936 (wild type) was grown and samples were analyzed by immunoblotting as described in Materials and Methods.

13 ± 7 nM at 2 h and 5 ± 5 nM at 6 h, which is <10% of the values found in LB medium alone (12).

The effect of glucose on *oxyR'::lacZ* expression was consistent with possible regulation by cAMP receptor protein. This hypothesis was tested more directly by introducing an adenylate cyclase (*cya*) mutation, which abolished expression of the single-copy *oxyR'::lacZ* fusion throughout the growth curve (Fig. 4A). In order to determine dependence of this effect on Crp, it was necessary to employ a multicopy plasmid containing the *oxyR'::lacZ* fusion (pAQ23), which was introduced into *cya* single-mutant and *cya crp* double-mutant strains. For the multicopy reporter, adenylate cyclase deficiency partially suppressed *oxyR'::lacZ* induction in late-exponential and stationary phases (Fig. 4B). The additional elimination of Crp almost eliminated the variation in *oxyR* expression during growth (Fig. 4B). The differences in the pattern of oscillation of *oxyR* expression observed for the multicopy plasmid in a wild-type strain (Fig. 4B) compared to that for the single-copy fusion (Fig. 1A) suggest some degree of disregulation with the multicopy reporter.

Global changes in gene expression patterns in *E. coli* can also be orchestrated by changes in RNA polymerase (26). Replacement of the σ^{70} subunit with σ^S (σ^{38}) alters RNA

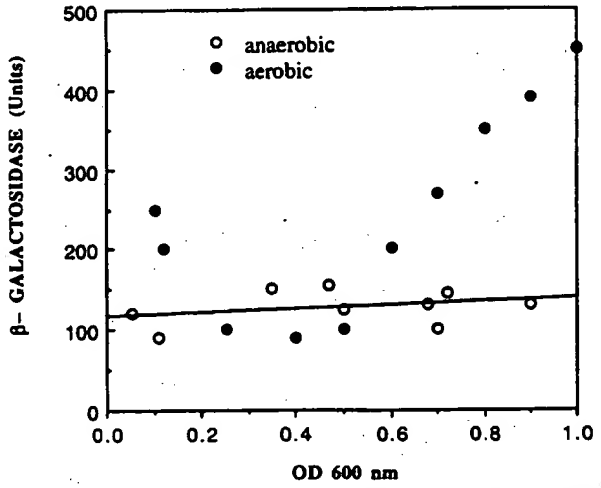

FIG. 2. Expression of a *oxyR'::lacZ* operon fusion in anaerobically growing *E. coli*. Cultures of strain BGF930 were grown at 37°C in 2-ml vials filled to the top with medium and fitted with butyl rubber stoppers (open circles). Data from aerobic experiments performed as described in the legend to Fig. 1 are shown for comparison. OD 600 nm, optical density at 600 nm.

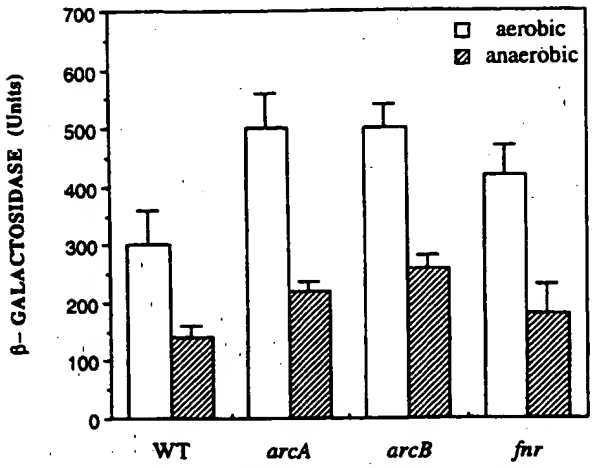

FIG. 3. Expression of a *oxyR'::lacZ* operon fusion in *arcAB* and *fnr E. coli*. Experimental conditions were as described in the legend to Fig. 2, with β-galactosidase activity determined at an optical density at 600 nm of 0.4 (~3 h of aerobic growth or ~16 h of anaerobic growth) for strains BGF940 (wild type [WT]), BGF950 (*fnr*), BGF960 (*arcB*), and BGF970 (*arcA*).

polymerase promoter specificity in stationary phase or upon starvation (19, 22). The *oxyR* promoter is a typical σ^{70} type (7), but the −35 consensus sequence for σ^S is not firmly established and could also be present. We therefore monitored expression of *oxyR* in an *rpoS* strain (BGF1030), a wild-type strain (BGF930), and a strain carrying a multicopy *rpoS*$^+$ (BGF930 carrying pDEB2). The expression of *oxyR* in the *rpoS* strain was the same as found for *rpoS*$^+$ cells during exponential growth but then increased steadily as the cells entered and remained in stationary phase (Fig. 5A). Thus, *oxyR* expression is maintained when there is no transition from σ^{70} to σ^S. Conversely, elevated expression of σ^S (*rpoS*) in exponential phase prevents the normal induction of *oxyR* (Fig. 5B). Elevated levels of functional σ^S in strains carrying pDEB2 are indicated by a ~10-fold-increased level f expressi n of the σ^S-regulated *bolA* gene during exponential growth (2).

Biological significance of the variati n in OxyR levels. To address the relevance of the changes in *oxyR* expression during exponential growth, we determined the expression of two activities under *oxyR* control, catalase hydroperoxidase I (HPI), and glutathione reductase, and the susceptibility f exponentially growing bacteria to killing by H_2O_2. β-Galactosidase activity from a single-copy *katG'::lacZ* operon fusion and glutathione reductase enzyme activity were significantly decreased in a Δ*oxyR* strain (BGF933), as expected, and in strains with continuously reduced *oxyR* expression. Both increased *rpoS* copy number and genetic deficiency in adenylate cyclase/Crp, diminished the expression of *katG'::lacZ* throughout growth (approximately twofold and up to fivefold, respectively; Table 2). The sensitivity to exogenously added H_2O_2 in strains with multicopy-*rpoS*$^+$ or *cya crp* mutations was increased to the level found for a Δ*oxyR* strain (Table 2). A similar hypersensitivity to H_2O_2 (relative killing zone, 1.5 ± 0.1) was found for the Δ*cya* single mutant (strain BGF612).

DISCUSSION

The results presented here show an oscillation in *oxyR* expression during aerobic growth in *E. coli*, including a pronounced increase in OxyR during early exponential phase. This induction was eliminated by *cya cpr* mutations, which also prevented the normal elevation of catalase and glutathione reductase activities in exponential phase and increased cellular sensitivity to hydrogen peroxide. The decreased expression f *oxyR* in stationary phase could be mimicked in exponentially growing cells by increased copy number of *rpoS*, encoding stationary-phase-induced σ^S protein, which also rendered the cells more sensitive to H_2O_2. These are the first examples f variations in the expression of *oxyR* and the first indication that such changes can affect the expression of OxyR-regulated genes.

Crp protein and cAMP regulate the synthesis of many inducible enzymes in *E. coli*. Catabolite repression is mediated

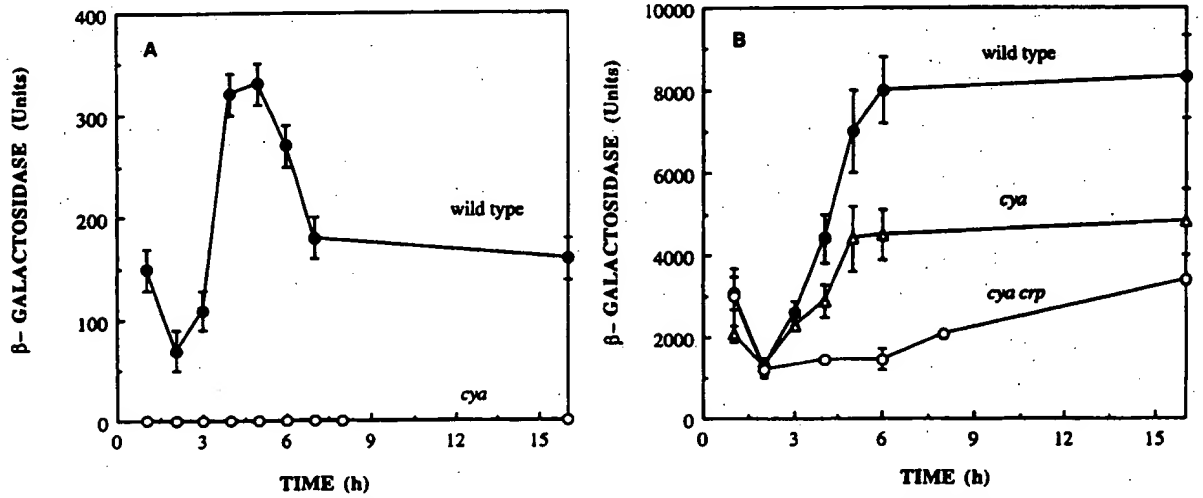

FIG. 4. (A) Expression of a single-copy *oxyR'::lacZ* operon fusion in an aerobically growing *E. coli cya* strain. Strains BGF930 and BGF612 were grown and sampled as described in the legend to Fig. 1. (B) Expression of a multicopy *oxyR'::lacZ* operon fusion in aerobically growing *E. coli cya* and *cya crp* strains. Cultures of strains CA8000 (wild type), CA8306 (*cya*), and CA8445 (*cya crp*) carrying plasmid pAQ23 (*oxyR'::lacZ*) grown overnight were diluted 100-fold in fresh LB broth and incubated at 37°C with shaking. Samples were taken periodically and assayed for β-galactosidase activity.

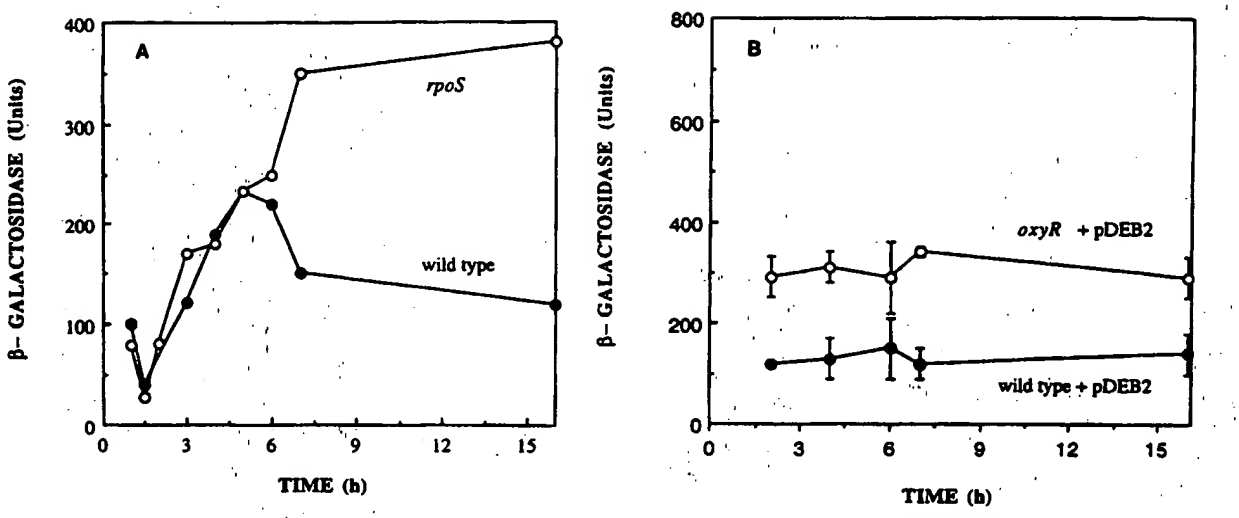

FIG. 5. Expression of a single-copy *oxyR'::lacZ* operon fusion in aerobically growing *E. coli* with altered *rpoS* expression. Experimental conditions were as described in the legend to Fig. 1A. (A) Strains BGF930 (*oxyR*$^+$; filled circles) and BGF1030 (*rpoS*) were grown, sampled, and assayed for β-galactosidase activity. (B) Strains BGF930 (*oxyR*$^+$) and BGF932 (*oxyR*) carrying plasmid pDEB2 (*rpoS*) were assayed for β-galactosidase expression during growth.

by a reduction in cAMP levels due to inhibition of adenylate cyclase by glucose. When the level of glucose decreases, the rise in intracellular cAMP concentration activates Crp to trigger gene expression (21). The catabolite repression reported two decades ago (14) for HPI, the product of the OxyR-dependent *katG* gene, now appears to be an indirect effect. Because the *katG* promoter has no known Crp-binding site, the Crp- or cAMP-dependent regulation of *oxyR* is the most likely mechanism to explain the glucose repression of catalase. Such indirect regulation would be expected for other OxyR-dependent genes. The loss of *oxyR* induction in a *cya crp* strain was accompanied by a 80% decrease in *katG* expression, a 55% decrease in glutathione reductase activity, and increased cellular sensitivity to H_2O_2 (Table 2), consistent with inadequate antioxidant defenses in these cells. One may speculate that catabolite repression of *oxyR* reflects the major contribution of respiration to the generation of intracellular hydrogen peroxide, the threat of which is diminished during growth in glucose.

The promoter selectivity of RNA polymerase is rapidly modulated by changes in the σ subunit (19, 22). RNA polymerase containing σ^{70} is the main form during exponential growth, while the enzyme containing σ^S increases upon some types of starvation or during the transition to stationary phase (19). The increase in the σ^S/σ^{70} ratio in stationary phase may be the key change affecting *oxyR*, since expression of *rpoS* from a multicopy plasmid also suppressed OxyR. The increased sensitivity to H_2O_2 resulting from *rpoS* overexpression is somewhat paradoxical, in that pronounced oxidant resistance is a hallmark of stationary-phase or starving *E. coli* (1, 18). It may be that the loss of the *oxyR* pathway is insufficiently compensated by the expression of *rpoS*-dependent genes under our conditions. In this regard, it should be noted that some genes (e.g., *dps*) (1) are controlled by both *oxyR* and *rpoS*.

The onset of aerobic exponential growth in *E. coli* is associated with a ~10-fold increase in the rate of H_2O_2 production from metabolic sources (11). Bacteria cope with this endogenous oxidative stress by increasing the OxyR-dependent transcription of the *katG*-encoded HPI (12). As a result of this homeostatic response, changes in the intracellular concentration of H_2O_2 are dampened to <2-fold (12). The Crp-dependent upregulation of *oxyR* reported here is evidently a key facet of the overall mechanism that acts to limit growth-dependent oxidative stress in *E. coli*. This regulation may be accounted for by the strongly increased cAMP levels reported for *E. coli* grown into stationary phase (34). Indeed, expression of *oxyR* was at least partly dependent on adenylate cyclase, although some other Crp-regulated promoters are not governed by cAMP levels (5). These include the *cst*-regulated genes which are induced in the stationary phase or under carbon starvati n (5), L-asparaginase (21), and the *gyrA* gene encoding a DNA gyrase subunit (10). The elucidation of the signaling pathway that initiates induction of *oxyR* merits further study. For example, possible correlation between cAMP levels and rates f endogenous H_2O_2 generation under different growth conditions should be determined.

TABLE 2. OxyR-dependent activities and sensitivity to oxidative stress in exponential-phase cultures of strains with different levels of *oxyR* expression[a]

Strain (relevant genotype)	*oxyR'::lacZ*	*katG'::lacZ*	GSSG[d] reductase	H_2O_2 sensitivity (relative diameter of killing zone)
oxyR$^+$[b]	1	1	1	1
Δ*oxyR*[c]	1.5 ± 0.1	0.30 ± 0.03	0.8 ± 0.1	1.4 ± 0.1
BGF931(pDEB2) (multicopy *rpoS*)	0.27 ± 0.01	0.60 ± 0.01	0.70 ± 0.05	1.7 ± 0.1
CA800 (*cya*$^+$ *crp*$^+$)	1	1	1	1
CA8445 (Δ*cya* Δ*crp*)	0.30 ± 0.01	0.20 ± 0.01	0.45 ± 0.05	1.3 ± 0.1

[a] Cultures grown overnight were diluted 1/100 and grown aerobically for 4 h. The results (means ± standard errors) are relative to the values for the corresponding wild-type strain.

[b] *oxyR::lacZ* level of expression was measured in strain BGF930; *katG'::lacZ*, GSSG reductase, and H_2O_2 sensitivity were measured in strain BGF931.

[c] *oxyR'::lacZ* level of expression was measured in strain BGF932; *katG'::lacZ*, GSSG reductase, and H_2O_2 sensitivity were measured in strain BGF933.

[d] 6556, oxidized glutathione.

ACKNOWLEDGMENTS

We are grateful to E. Martins and E. Hidalgo and other members of the laboratory for advice and discussions. We thank G. Storz for helpful discussions and for providing us with strain TA4112, plasmids pAQ17 and pAQ23, and antibodies against OxyR; R. Gunsalus for providing strain RK5288; E. Brickman for providing strains CA8000 and CA8445; R. Kolter for providing plasmid pDEB2 and strain ZK1001; E. C. C. Lin for providing strains ECL594 and ECL906; A. Hochschild for providing strain M7141; and A. S. Lynch for helpful discussion and for performing DNA binding assays with ArcA.

This work was supported by National Institutes of Health (CA37831) and the Amyotrophic Lateral Sclerosis Association.

REFERENCES

1. **Altuvia, S., M. Almiron, G. Hulsman, R. Kolter, and G. Storz.** 1994. The *dps* promoter is activated by OxyR during growth and by IHF and σ^S in stationary phase. Mol. Microbiol. 13:265–272.
2. **Bohannon, D. E., N. Connell, J. Keener, A. Tormo, M. Espinosa-Urgel, M. M. Zambrano, and R. Kolter.** 1991. Stationary-phase-inducible "gearbox" promoters: differential effects of *katF* mutations and role of σ^{70}. J. Bacteriol. 173:4482–4492.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113.
4. **Brickman, J., J. Soll, and J. Beckwith.** 1973. Genetic characterization of mutations which affect catabolite-sensitive operons in *Escherichia coli*, including deletions of the gene for adenylate cyclase. J. Bacteriol. 116:582–587.
5. **Busby, S., and A. Kolb.** 1996. The Cap modulon, p. 255–279. *In* E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. R. G. Landes Company, Austin, Tex.
6. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell 41:753–762.
7. **Christman, M. F., G. Storz, and B. Ames.** 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA 86:3484–3488.
8. **Demple, B., and J. Halbrook.** 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature 304:466–468.
9. **Emory, S. A., and J. C. Belasco.** 1990. The *ompA* 5′ untranslated RNA segment functions in *Escherichia coli* as a growth-rate-regulated mRNA stabilizer whose activity is unrelated to translational efficiency. J. Bacteriol. 172:4472–4481.
10. **Gomez-Gomez, J. M., F. Baquero, and J. Blazquez.** 1996. Cyclic AMP receptor protein positively controls *gyrA* transcription and alters DNA topology after nutritional upshift in *Escherichia coli*. J. Bacteriol. 178:3331–3334.
11. **González-Flecha, B., and B. Demple.** 1995. Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. J. Biol. Chem. 270:13681–13687.
12. **González-Flecha, B., and B. Demple.** 1997. Homeostatic regulation of the intracellular concentration of hydrogen peroxide in aerobically growing *Escherichia coli*. J. Bacteriol. 179:382–388.
13. **Greenberg, J., and B. Demple.** 1989. A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. J. Bacteriol. 171:3933–3939.
14. **Hassan, H. M., and I. Fridovich.** 1978. Regulation of the synthesis of catalase and peroxidase in *Escherichia coli*. J. Biol. Chem. 253:6445–6450.
15. **Hidalgo, E., and B. Demple.** 1996. Adaptive responses to oxidative stress: the *soxRS* and *oxyR* regulons, p. 435–452. *In* E. C. C. Lin and A. S. Lynch (ed.), Regulation of gene expression in *Escherichia coli*. R. G. Landes Company, Austin, Tex.
16. **Iuchi, S., D. Furlong, and E. C. Lin.** 1989. Differentiation of *arcA*, *arcB*, and *cpxA* mutant phenotypes of *Escherichia coli* by sex pilus formation and enzyme regulation. J. Bacteriol. 171:2889–2893.
17. **Iuchi, S., A. Aristarkhov, J. M. Dong, J. S. Taylor, and E. C. C. Lin.** 1994. Effects of nitrate respiration on expression of the Arc-controlled operons encoding succinate dehydrogenase and flavin-linked L-lactate dehydrogenase. J. Bacteriol. 176:1695–1701.
18. **Jenkins, D. E., J. E. Shultz, and A. Martin.** 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. J. Bacteriol. 170:3910–3914.
19. **Jishage, M., and A. Ishihama.** 1995. Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of σ^{70} and σ^{38}. J. Bacteriol. 177:6832–6835.
20. **Jones, H. M., and R. P. Gunsalus.** 1987. Regulation of *Escherichia coli* fumarate reductase (*frdABCD*) operon expression by respiratory electron acceptors and the *fnr* gene product. J. Bacteriol. 169:3340–3349.
21. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. 62:740–795.
22. **Losick, R.** 1994–1995. RNA polymerase sigma factors and cell-specific gene transcription in a simple developing organism. Harvey Lect. 90:1–17.
23. **Lynch, A. S.** Personal communication.
24. **Miller, J. H.** 1992. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. Ames.** 1986. Hydrogen-peroxide inducible proteins in *Salmonella typhimurium* overlap with heat shock and other proteins. Proc. Natl. Acad. Sci. USA 83:8059–8063.
26. **Reznikoff, W. S., D. A. Siegele, D. W. Cowing, and C. Gross.** 1985. The regulation of transcription initiation in bacteria. Annu. Rev. Genet. 19:355–387.
27. **Sabourn, D., and J. Beckwith.** 1975. Deletion of the *Escherichia coli crp* gene. J. Bacteriol. 122:338–340.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene 53:85–96.
30. **Storz, G., L. A. Tartaglia, and B. N. Ames.** 1990. Transcriptional regulation of oxidative stress-inducible genes: direct activation by oxidation. Science 248:189–194.
31. **Tartaglia, L. A., G. Storz, and B. N. Ames.** 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. J. Mol. Biol. 210:709–719.
32. **Triggs-Raine, B. L., and P. C. Loewen.** 1987. Physical characterization of *katG*, encoding catalase HPI of *Escherichia coli*. Gene 52:121–128.
33. **Twigg, A. J., and D. Sherratt.** 1980. Trans-complementable copy-number mutants of plasmid ColE1. Nature 283:216–218.
34. **Ullman, A., and A. Danchin.** 1983. Role of cyclic AMP in bacteria. Adv. Cyclic Nucleotide Res. 15:3–53.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.

Mol Gen Genet (1989) 218:371–376

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Molecular cloning and nucleotide sequencing of *oxyR*, the positive regulatory gene of a regulon for an adaptive response to oxidative stress in *Escherichia coli:* Homologies between OxyR protein and a family of bacterial activator proteins

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Summary. Treatment of *Escherichia coli* and *Salmonella typhimurium* cells with a low dose of hydrogen peroxide induces expression of a large number of genes, and confers resistance to oxidative stresses. The *oxyR* gene encodes a positive regulatory protein for a subset of these genes involved in the defense against oxidative damage. We cloned a DNA fragment that contains the *E. coli oxyR* region on a plasmid vector, and analyzed the nucleotide sequence of the gene. The amino acid sequence of OxyR protein, deduced from the nucleotide sequence, shows a high degree of homology to the sequences of a number of bacterial activator proteins including LysR, CysB, IlvY, MetR and NodD. The product of the *oxyR* gene identified by the maxicell procedure was a 34 kDa protein, which agrees with the size predicted from the nucleotide sequence of the gene.

Key words: Oxidative stress – Activator protein – *oxyR* – LysR family

Introduction

Reactive oxygen species such as hydrogen peroxide, superoxide anion, singlet oxygen and hydroxy radical are generated in vivo during normal aerobic metabolism as well as during exposure to ionizing radiation. They damage DNA, protein and membrane lipids. Cytotoxic and mutagenic effects of oxygen are attributed to these reactive oxygen species (Fridovich 1978).

Escherichia coli has defense mechanisms against the toxicity of these reactive oxygen species. First, there are enzymes which scavenge them, such as catalases and superoxide dismutases. Furthermore, there are DNA repair systems for oxidatively damaged DNA. These systems involve DNA polymerase I, exonuclease III, endonuclease IV, RecA and RecBC proteins (Linn and Imlay 1987).

In *E. coli* and *Salmonella typhimurium*, pretreatment of exponentially growing cells with sublethal levels of hydrogen peroxide makes cells resistant to subsequent exposure to higher concentrations of hydrogen peroxide and other stresses such as ionizing radiation, near UV, heat, and a variety of chemical oxidants (Demple and Halbrook 1983; Christman et al. 1985; Tyrrell 1985). The acquisition of resistance involves induction of both reactive oxygen scavenging systems (Winquist et al. 1984) and DNA repair systems which are distinct from either SOS systems or the adaptive response to alkylating agents (Demple and Halbrook 1983).

The *oxyR* gene is a positive regulatory gene for at least nine proteins that are induced by treatment with hydrogen peroxide in *S. typhimurium* (Christman et al. 1985). These proteins are catalase/hydroperoxidase I, glutathione reductase, alkylhydroperoxide reductase, and proteins whose functions are unidentified (Christman et al. 1985). *oxyR* mutants are hypersensitive to oxidative stresses and highly mutable in aerobic growth conditions (Storz et al. 1987; Greenberg and Demple 1988), indicating the importance of the *oxyR* regulated system for aerobic growth.

To understand the functions of the genes involved in the defense against oxidative stresses, and to elucidate regulatory mechanisms of the expression of these genes, we cloned the related genes. In this paper we report the cloning of a key gene for regulation, *oxyR*, and the nucleotide sequence of the gene. The primary structure of OxyR protein, deduced from the nucleotide sequence, revealed a high degree of homology to the sequences of a large family of bacterial activator proteins.

Materials and methods

Bacterial strains, phages, plasmids and media. E. coli strains RK4936 (*oxyR*$^+$ *butB*::Tn*10*) and TA4112 (as RK4936 but *Δ*[*oxyR-btuB*]) (Christman et al. 1985) were obtained from B.N. Ames. CSR603 (*recA*1, *uvrA*6, *phr*-1) (Sancar et al. 1981) and JM103 (*Δ*[*pro-lac*], *supE*, *thi*/F′*traD*36, *proAB*, *lacI*^{q}*ZΔ*M15) (Messing et al. 1981) were used for the maxicell analysis and as a host strain for M13 phages, respectively.

pACYC184 (Chang and Cohen 1978), pBR322 (Bolivar et al. 1977), pUC18 and pUC19 (Yanisch-Perron et al. 1985) were used as cloning vectors. pKT1033, a derivative of low copy plasmid pMF3 (Manis and Kline 1977), carries a *katG′–′lacZ* fusion gene (details to be published elsewhere). M13mp8 and M13mp9 (Messing and Vieira 1982) were used for DNA sequencing. An *E. coli* gene library was obtained from Y. Kohara (Kohara et al. 1987).

LB medium was used for routine growth of bacteria. M9 medium supplemented with appropriate amino acids was used for labeling the cells by the maxicell procedure. YT and 2YT medium were used for M13 phages. All these media were described by Miller (1972).

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Recombinant DNA techniques. Preparati n and in vitro manipulation of DNA were d ne by standard procedures as described (Maniatis et al. 1982).

DNA sequencing. The DNA fragment carrying the *oxyR* regi n was subcloned into M13mp8 and M13mp9. Series of deletions from ne end f the cl ned DNA fragment were generated by the method of H ng (1982). The nucleotide sequence was determined by the dideoxy chain-termination method (Sanger et al. 1977) employing a series of M13 phage derivatives.

Identification of plasmid encoded proteins by the maxicell procedure. The proteins encoded by plasmids were analyzed after labeling the cells with [^{35}S]-methionine by the maxicell procedure of Sancar et al. (1981). Proteins were resolved by electrophoresis in a 15% polyacrylamide gel containing sodium dodecylsulfate (SDS-PAGE) according to Laemmli (1970) and visualized by autoradiography.

Hydrogen peroxide sensitivity assay. Exponentially growing cells were diluted appropriately and plated onto LB agar plates containing various concentrations of hydrogen peroxide. Colonies were counted after incubation for 24 h at 37° C.

Assay of β-galactosidase activity. β-galactosidase activity was assayed in exponentially growing cells harboring pKT1033 cultured in the presence or absence of 100 μM hydrogen peroxide for the indicated period. Procedures for the assay and calculation of the activity were as described by Miller (1972).

Results

Cloning of the oxyR *gene*

Since *oxyR* was mapped at 90 min on the *E. coli* genetic map (Christman et al. 1985), we used the ordered genomic library and physical map of the whole *E. coli* chromosome constructed by Kohara et al. (1987) to clone the *oxyR* gene. DNAs of recombinant phages which carry the chromosomal DNA fragments around 90 min on the *E. coli* chromosome were isolated. After digestion of the recombinant DNAs with appropriate restriction enzymes according to the physical map, DNA fragments were cloned into multicopy plasmid vectors. These hybrid plasmids were tested for their ability to complement hydrogen peroxide sensitivity of TA4112 (*ΔoxyR*). Among the plasmids constructed, one plasmid, pKT5352 (Fig. 1), which carries the 11.5 kb *Eco*RI fragment of 4G11 (Kohara et al. 1987) on pACYC184, conferred hydrogen peroxide resistance to the *ΔoxyR* mutant (Fig. 2).

oxyR is a positive regulatory gene for *katG* which encodes catalase/hydroperoxidase I in *S. typhimurium* (Morgan et al. 1986). Using a *katG'—'lacZ* fusion gene carried on pKT1033, we examined the *oxyR* dependence of the induction of *katG* in *E. coli* (Fig. 3). A *ΔoxyR* strain TA4112 did not induce *katG* expression by hydrogen peroxide as in the case of the *ΔoxyR* strain of *S. typhimurium*. β-galactosidase activity encoded by the fusion gene was, however, induced in TA4112 harboring pKT5352, as in the parental *oxyR*$^+$ strain, in response to a low concentration of hydrogen peroxide. These results strongly suggest that

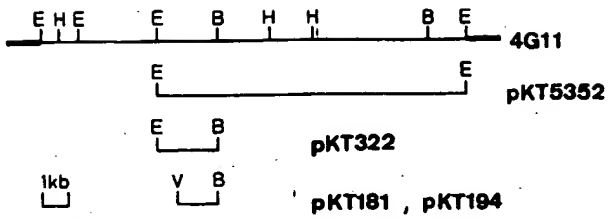

Fig. 1. Restriction cleavage map of the *Escherichia coli* chromosomal DNA fragment containing the *oxyR* gene. The top line illustrates the region carried on 4G11 (Kohara et al. 1987). The lines below indicate the regions of DNA subcloned into the plasmid vectors. Abbreviations of restriction enzymes are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; V, *Eco*RV

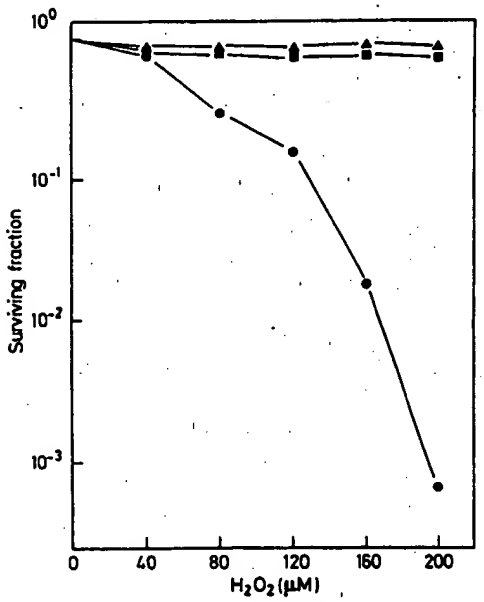

Fig. 2. Complementation of hydrogen peroxide sensitivity of an *oxyR* mutant TA4112 by pKT5352. Exponentially growing cultures of TA4112/pACYC184 (●), TA4112/pKT5352 (▲) and RK4936/pACYC184 (■) were plated on LB plates containing various concentrations of hydrogen peroxide and were incubated overnight for colony formation

the chromosomal DNA segment carried on pKT5352 contains the *oxyR* gene.

Subclonig of the oxyR *gene*

To locate the *oxyR* gene more precisely, smaller fragments were subcloned and tested for their ability to complement the *oxyR* mutation by examining hydrogen peroxide sensitivity (Fig. 1). Using *Bam*HI and *Hin*dIII restriction enzyme recognition sites (Fig. 1), subfragments were cloned into pBR322. The hybrid plasmid pKT322 that contains the 2.4 kb *Eco*RI—*Bam*HI fragment complemented the *oxyR* mutation. This fragment was further cleaved by *Eco*RV and the subfragments were cloned into pUC18 and pUC19. The hybrid plasmids pKT181 and pKT194 that contain the 1.5 kb *Eco*RV—*Bam*HI fragment in the *Hin*cII—*Bam*HI sites (Fig. 1) complemented the *oxyR* mutation.

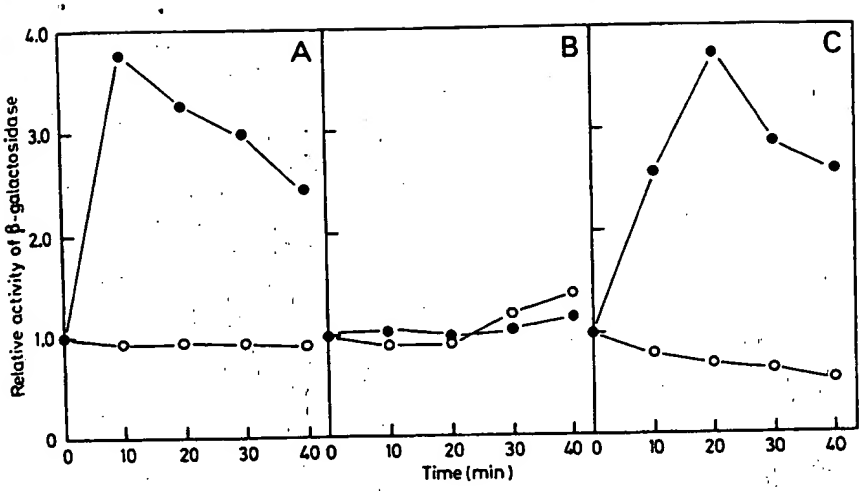

Fig. 3A–C. Induction of β-galactosidase activity encoded by a *katG'–'lacZ* fusion gene. Overnight cultures of RK4936/pACYC184/pKT1033 (A), TA4112/pACYC184/pKT1033 (B) and TA4112/pKT5352/pKT1033 (C) were diluted 100-fold with fresh medium, and were grown to a density of 10^8 cells per ml. At time zero, hydrogen peroxide (100 μM) was added to one portion of each culture. Samples were taken from hydrogen peroxide treated (•) and untreated (o) cultures, and β-galactosidase activity was measured

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GGATCCTGGAGATCCGCAAAAGTTCACGTT GGCTTTAGTTATTCGAGTTGAGAAACTCTC GAAACGGGCAGTGACTTCAAGGGTTAAAAG    90

AGGTGCCGCTCCGTTTCTGTGAGCAATTAT CAGTCAGAATGCTTGATAGGGATAATCGTT CATTGCTATTCTACCTATCGCCATGAACTA   180

TCGTGGCGATGGAGGATGGATAATGAATAT TCGTGATCTTGAGTACCTGGTGGCATTGGC TGAACACCGCCATTTTCGGCGTGCGGCAGA   270
                    MetAsnIle ArgAspLeuGluTyrLeuValAlaLeuAla GluHisArgHisPheArgArgAlaAlaAsp

TTCCTGCCACGTTAGCCAGCCGACGCTTAG CGGGCAAATTCGTAAGCTGGAAGATGAGCT GGGCGTGATGTTGCTGGAGCGGACCAGCCG   360
 SerCysHisValSerGlnProThrLeuSer GlyGlnIleArgLysLeuGlnAspGluLeu GlyValMetLeuLeuGluArgThrSerArg

TAAAGTGTTGTTCACCCAGGCGGGAATGCT GCTGGTGGATCAGGCGCGTACCGTGCTGCG TGAGGTGAAAGTCCTTAAAGAGATGGCAAG   450
 LysValLeuPheThrGlnAlaGlyMetLeu LeuValAspGlnAlaArgThrValLeuArg GluValLysValLeuLysGluMetAlaSer

CCAGCAGGGCGAGACGATGTCCGGACCGCT GCACATTGGTTTGATTCCCACAGTTGGACC GTACCTGCTACCGCATATTATCCCTATGCT   540
 GlnGlnGlyGluThrMetSerGlyProLeu HisIleGlyLeuIleProThrValGlyPro TyrLeuLeuProHisIleIleProMetLeu

GCACCAGACCTTTCCAAAGCTGGAAATGTA TCTGCATGAAGCACAGACCCACCAGTTACT GGCGCAACTGGACAGCGGCAAACTCGATTG   630
 HisGlnThrPheProLysLeuGluMetTyr LeuHisGluAlaGlnThrHisGlnLeuLeu AlaGlnLeuAspSerGlyLysLeuAspCys

CGTGATCCTCGCGCTGGTGAAAGAGAGCGA AGCATTCATTGAAGTGCCGTTGTTTGATGA GCCAATGTTGCTGGCTATCTATGAAGATCA   720
 ValIleLeuAlaLeuValLysGluSerGlu AlaPheIleGluValProLeuPheAspGlu ProMetLeuLeuAlaIleTyrGluAspHis

CCCGTGGGCGAACCGCGAATGCGTACCGAT GGCCGATCTGGCAGGGGAAAAACTGCTGAT GCTGGAAGATGGTCACTGTTTGCGCGATCA   810
 ProTrpAlaAsnArgGluCysValProMet AlaAspLeuAlaGlyGluLysLeuLeuMet LeuGluAspGlyHisCysLeuArgAspGln

GGCAATGGGTTTCTGTTTTGAAGCCGGGGC GGATGAAGATACACACTTCCGCGCGACCAG CCTGGAAACTCTGCGCAACATGGTGGCGGC   900
 AlaMetGlyPheCysPheGluAlaGlyAla AspGluAspThrHisPheArgAlaThrSer LeuGluThrLeuArgAsnMetValAlaAla

AGGTAGCGGGATCACTTTACTGCCAGCGCT GGCTGTGCCGCCCGGAGCGCAAACGCGATGG GGTTGTTTATCTGCCGTGCATTAAGCCGGA   990
 GlySerGlyIleThrLeuLeuProAlaLeu AlaValProProGluArgLysArgAspGly ValValTyrLeuProCysIleLysProGlu

ACCACGCCCGCACTATTGGCCTGGTTTATCG TCCTGGCTCACCGCTGCGCAGCCGCTATGA GCAGCTGGCAGAGGCCATCCGCGCAAGAAT- 1080
 ProArgArgThrIleGlyLeuValTyrArg ProGlySerProLeuArgSerArgTyrGlu GlnLeuAlaGluAlaIleArgAlaArgMet

GGATGGCCATTTCGATAAAGTTTTAAAACA GGCGGTTTAAACCGTTTAACGCAGCTACCC GATAGGCTTCCGCCATCGTCGGGTAGTTAA 1170
 AspGlyHisPheAspLysValLeuLysGln AlaVal***

AGGTGGTGTTGACGAAGTACTCAATAGTGT TGCCGCCACCTTTCTGTTCCATAATCGCCT GACCGATATGAATAATTTCGGCAGCGCGCT 1260

CGCCAAAGCAGTGAATACCCAGAATCTCTT TTGTTTCCCGATGGAACAAAATTTTCAGCG TGCCCACGTTCATGCCGACGATTTGTGCGC 1350

GTGCCAGATGTTTAAACTGGGCGCGGCCCA CTTCATATGGCACTTTCATTGCGGTCAGCT GCTGTTCGGTTTTGCCCACAGAGCTGATTT 1440

CCGGGATGGTGTAAATACCGGTAGGGATAT C
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Fig. 4. Nucleotide sequence of the *oxyR* gene. The deduced amino acid sequence is also shown. The putative −10 and −35 sequences are *underlined*. The Shine-Dalgarno sequence is *boxed*. A region of dyad symmetry is indicated by *inverted arrows*

DNA sequence of the oxyR *region*

The 1.5 kb *Eco*RI–*Hin*dIII fragment of pKT194 was subcloned into the *Eco*RI–*Hin*dIII sites of M13mp8 and M13mp9, and series of deletions from one end into the inserted fragment were generated by the method of Hong (1982). The DNA sequence of the *oxyR* region was analyzed by the dideoxy chain-termination method using these deletion phage clones.

One long open reading frame that could code for a 34 kDa polypeptide was found (Fig. 4). There were two potential translation initiation codons at the beginning of this reading frame (positions 173 and 203). The ATG codon at nucleotide 203 was preceded by a typical Shine-Dalgarno

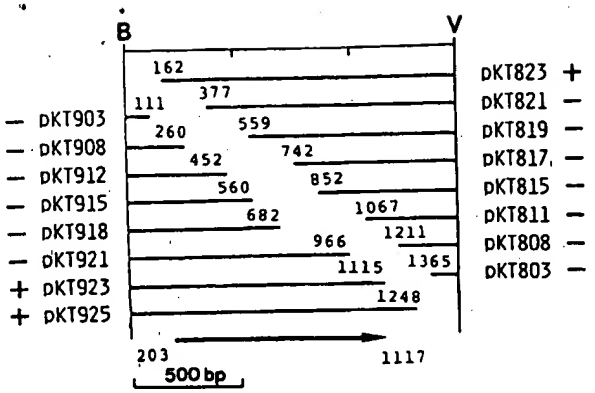

Fig. 5. Complementing ability of the deletion derivatives of pKT181 and pKT194. The regions which are carried on the plasmids are shown as *horizontal lines*, and the deletion endpoints are indicated by the sequence numbers used in Fig. 4. The *bottom arrow* indicates the extent and direction of the *oxyR* coding region. B and V indicate the restriction enzyme sites for *Bam*HI and *Eco*RV, respectively. Complementing ability is indicated by + or − beside the plasmid number

sequence, GGAGG (Shine and Dalgarno 1974), but such a sequence was not found upstream of the potential initiation codon at nucleotide 173. Therefore, we think that the ATG codon at nucleotide 203 is the initiation site for translation. These data suggest that the coding region of *oxyR* is from nucleotide 203 to 1120, coding for a protein with 305 amino acids. Upstream of the *oxyR* coding region, the sequences substantially homologous to the consensus sequences for the −35 and −10 regions of *E. coli* promoters (McClure 1985) were found at nucleotides 133–138 (TTGAtA) and 157–162 (TATtcT), respectively. The distance between the two regions (18 bp) is also typical for promoters. Therefore, these regions likely constitute the promoter of *oxyR*. Downstream of the coding region, a DNA sequence with dyad symmetry followed by a T-rich sequence was found between nucleotides 1289 and 1315. This structure is typical for a rho-independent transcriptional terminator (Platt 1986) and, therefore, it may function as the terminator for *oxyR*.

Identification of the oxyR *gene product*

Several fragments with deleti ns in the *oxyR* region were excised from the M13 phage DNAs used for sequencing by digesting them at the flanking restriction sites with *Eco*RI and *Hin*dIII, and recloned int the same sites on pUC18 or pUC19. These recombinant plasmids were introduced into TA4112 (*ΔoxyR*) to test for complementing ability (Fig. 5). In the deletion series from the *Bam*HI site, the OxyR$^+$ phenotype was lost when the deletion extended to 377 bp. In the other deletion series, a 505 bp deletion from the *Eco*RV site (pKT921) inactivated *oxyR* function. pKT923 that is lacking the last codon of the gene sh wed an OxyR$^+$ phenotype, indicating that the last amino acid of the OxyR protein may not be essential for OxyR function.

The proteins encoded by these deletion plasmids were labeled with [^{35}S]-methionine by the maxicell procedure and analyzed by SDS-PAGE (Fig. 6). The cells carrying pKT194 synthesized a 34 kDa protein in addition to the 30 kDa β-lactamase encoded by the *bla* gene on the vector (Fig. 6B, lane 1). Derivatives of pKT194, pKT821 and pKT819, in which *oxyR* was inactivated by deletion fr m the *Bam*HI site expressed no protein other than β-lactamase (Fig. 6B, lanes 3, 4). In the case of deletions from the *Eco*RV site, parental plasmid pKT181 and its deleti n derivative pKT925, which is OxyR$^+$, synthesized the 34 kDa protein and the 30 kDa β-lactamase (Fig. 6A, lanes 2, 3). Since the 34 kDa protein was synthesized by the OxyR$^+$ plasmids but not by the OxyR$^-$ plasmids, and the size f the protein agrees with the one predicted from the nucleotide sequence of *oxyR*, we concluded that it is the product of *oxyR*. Smaller polypeptides, probably truncated forms of the 34 kDa protein and their degradation products, were observed in the cells containing OxyR$^-$ deletion plasmids (Fig. 6A, lanes 4, 5, 6). The sizes of the truncated peptides encoded by the deletion plasmids agree with the predicted sizes from the open reading frame of *oxyR*. These results further support the conclusion that the 34 kDa protein is

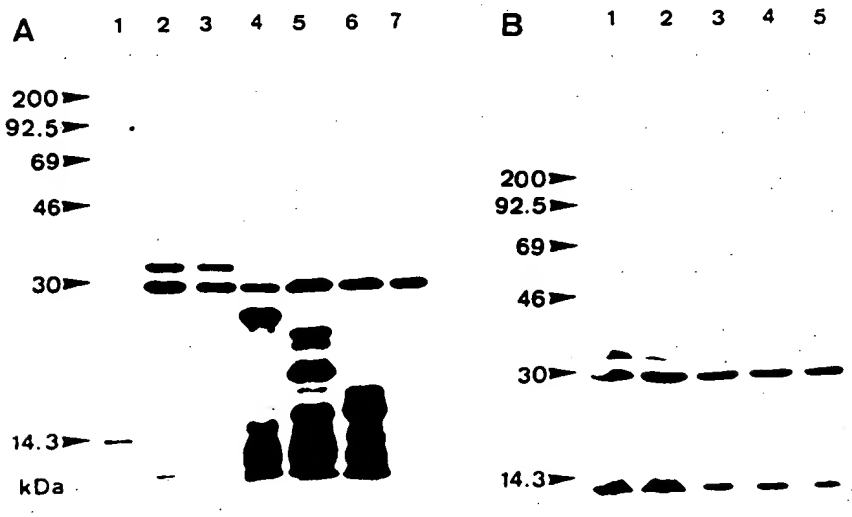

Fig. 6A and B. Identification of the *oxyR* gene product. Proteins produced in maxicells carrying the plasmids with deletions from the *Eco*RV (A) and *Bam*HI (B) sites, and their parental plasmids were analyzed in 15% SDS-polyacrylamide gels.
A Lane 1, ^{14}C-labeled molecular weight markers; 2, pKT181; 3, pKT925; 4, pKT918; 5, pKT915; 6, pKT912; 7, pUC18.
B Lane 1, pKT194; 2, pKT823; 3, pKT821; 4, pKT819; 5, pUC19

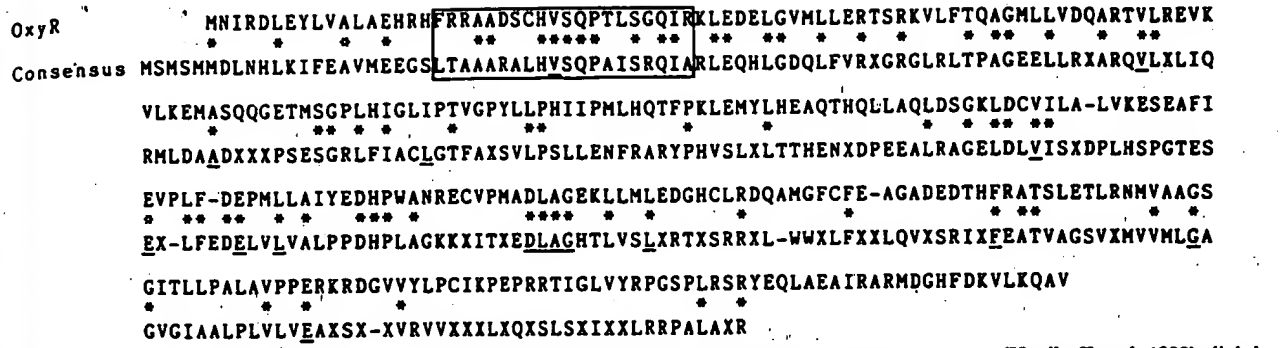

Fig. 7. Comparison of the amino acid sequence of OxyR protein with the LysR family consensus sequence (Henikoff et al. 1988) slightly modified by including the OxyR sequence. The consensus amino acids modified here are *underlined*. Identical residues are indicated by *asterisks*. The region of the predicted helix-turn-helix motif is *boxed*

the product of *oxyR* and the open reading frame shown in Fig. 4 is the coding region of the gene.

Homology between OxyR and other proteins

Homologies were searched for between the amino acid sequence of the OxyR protein deduced from the nucleotide sequence and sequences of other proteins in current databases. We found high degrees of homology (approximately 25%) with several *E. coli* positive regulatory proteins. They include CysB (Ostrowski et al. 1987), IlvY (Wek and Hatfield 1986) and LysR (Stragier and Patte 1983). Highly homologous regions among these proteins were found in the N-terminal halves, although some homologies were observed throughout the proteins. Recently, Henikoff et al. (1988) reported a large family of bacterial activator proteins which include the activators for the operons involved in amino acid biosynthesis. We compared the deduced sequence of OxyR protein with those of the nine proteins in this family, and the consensus sequence of nine proteins reported by them was slightly modified by including the OxyR sequence (Fig. 7). The sequence of the OxyR protein is highly homologous to the consensus sequence and, therefore, it belongs to the family.

In the N-terminal highly homologous region, the sequence of 20 amino acids of OxyR protein (residues 18–37) is similar to a helix-turn-helix motif of DNA binding proteins (Pabo and Sauer 1984) (Fig. 7). By the method for detection of potential λcro-like DNA binding region in proteins developed by Dodd and Egan (1987), a relatively high score (1523) was calculated for the OxyR protein in this region. Statistical analysis of about 2500 proteins, including both DNA-binding and non-binding proteins, indicates that proteins with a score above 1400 have a high probability of containing the DNA-binding region (Dodd and Egan 1987). This region aligns with the predicted helix-turn-helix consensus of the family. These results suggest that the N-terminal region of OxyR is involved in DNA binding, similarly to those of the other nine members of the family (Henikoff et al. 1988).

Discussion

We have cloned the DNA fragment from the *E. coli* chromosomal DNA library that complemented hydrogen peroxide sensitivity of an *oxyR* deletion mutant. Since this DNA fragment was located at 90 min where *oxyR* maps, and introduction of the plasmid carrying the DNA fragment recovered OxyR$^+$ phenotypes in the *oxyR* deletion mutant, as examined by sensitivity to hydrogen peroxide and inducibility of *katG* expression, we concluded that the cloned chromosomal DNA carried the *oxyR* gene. The size of the *oxyR* region that could function was determined to be about 0.9 kb, by constructing a series of deletion plasmids and analyzing the complementing ability of these plasmids. Since a 34 kDa protein was synthesized in maxicells carrying the OxyR$^+$ plasmids, which agrees with the open reading frame shown (Fig. 4), we think that the protein is the product of *oxyR* and the open reading frame is the coding region of the gene. Typical sequences for promoters and terminators were identified upstream and downstream of the coding region, respectively, so the gene may constitute a single cistronic operon.

OxyR protein is a positive regulator for a number of genes induced by oxidative stresses and, consistent with this, an homology search revealed that the protein is homologous to a large family of bacterial activator proteins. OxyR protein, similarly to other member of this family, possesses a sequence characteristic of a DNA-binding domain, and therefore it may bind to a unique consensus sequence in the regulatory regions of the genes under control, including *katG*, to activate transcription. To study the molecular mechanism of transcriptional regulation, we recently overproduced and purified OxyR protein. The sequence of the amino-terminal ten amino acids of the purified protein agreed with that deduced from the nucleotide sequence of *oxyR* (unpublished result). We are currently studying the interaction of the protein with the regulatory regions of *katG* and *oxyR*.

Acknowledgments. We thank B.N. Ames and Y. Kohara for kind gifts of bacterial strains and an *E. coli* gene library, respectively. We also thank M. Kato for encouragement, and T. Wada for help in computer analysis. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

Bolivar F, Rodriguez RL, Green PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, Falkow S (1977) Construction and characterization of new cloning vehicles. II, A multipurpose cloning system. Gene 2: 95–113

Chang ACY, Cohen SN (1978) Construction and characterization

of amplifiable multicopy cloning vehicles derived from the p15A cryptic miniplasmid. J Bacteriol 134:1141–1156

Christman MF, Morgan RW, Jacobson FS, Ames BN (1985) Positive control of a regulon for a defense against oxidative stress and heat-shock proteins in *Salmonella typhimurium*. Cell 41:753–762

Demple B, Halbrook J (1983) Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature 304:466–468

Dodd IB, Egan JB (1987) Systematic method for the detection of potential λcro-like DNA-binding regions in proteins. J Mol Biol 194:557–564

Fridovich I (1978) The biology of oxygen radicals. Science 201:875–880

Greenberg JT, Demple B (1988) Overproduction of peroxide-scavenging enzymes in *Escherichia coli* suppresses spontaneous mutagenesis and sensitivity to redox-cycling agents in *oxyR*⁻ mutants. EMBO J 8:2611–2617

Henikoff S, Haughn GW, Calou JM, Wallace JC (1988) A large family of bacterial activator proteins. Proc Natl Acad Sci USA 85:6602–6606

Hong GF (1982) A systematic DNA sequencing strategy. J Mol Biol 158:539–549

Kohara Y, Akiyama K, Isono K (1987) The physical map of whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting a large genomic library. Cell 50:495–508

Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Linn S, Imlay JA (1987) Toxicity, mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. J Cell Sci Suppl 6:289–301

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Manis JJ, Kline BC (1977) Restriction endonuclease mapping and mutagenesis of the F sex factor replication region. Mol Gen Genet 152:175–182

McClure WR (1985) Mechanism and control of transcription initiation in procaryotes. Annu Rev Biochem 54:171–204

Messing J, Vieira J (1982) A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene 19:269–276

Messing J, Crea R, Seeburg PH (1981) A system for shotgun DNA sequencing. Nucleic Acids Res 9:309–321

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Morgan RW, Christman MF, Jacobson FS, Storz G, Ames BN (1986) Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc Natl Acad Sci USA 83:8059–8063

Ostrowski J, Jagura-Burdzy G, Kredich NM (1987) DNA sequence of the *cysB* region of *Salmonella typhimurium* and *Escherichia coli*. J Biol Chem 262:5999–6005

Pabo CO, Sauer RT (1984) Protein-DNA recognition. Annu Rev Biochem 53:293–321

Platt T (1986) Transcription termination and regulation of gene expression. Annu Rev Biochem 55:339–372

Sancar A, Wharton RP, Seltzer S, Kaciniski BM, Clarke ND, Rupp WD (1981) Identification of the UvrA gene product. J Mol Biol 148:45–62

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Shine J, Dalgarno L (1974) The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosomal binding sites. Proc Natl Acad Sci USA 71:1342–1346

Storz G, Christman MF, Sies H, Ames BN (1987) Spontaneous mutagenesis and oxidative domage to DNA in *Salmonella typhimurium*. Proc Natl Acad Sci USA 84:8917–8921

Stragier P, Patte JC (1983) Regulation of diaminopimelate decarboxylase synthesis in *Escherichia coli*. III Nucleotide sequence and regulation of *lysR* gene. J Mol Biol 168:333–350

Tyrrell RM (1985) A common pathway for protection of bacteria against damage by solar UVA (334 nm, 365 nm) and an oxidizing agent (H_2O_2). Mutat Res 145:129–136

Wek RC, Hatfield GW (1986) Nucleotide sequence and in viv expression of the *ilvY* and *ilvC* genes in *Escherichia coli*. J Biol Chem 261:2441–2450

Winquist L, Rannug U, Rannug A, Ramel C (1984) Protection from toxic and mutagenic effects of H_2O_2 by catalase induction in *Salmonella typhimurium*. Mutat Res 141:145–147

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains; nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33:103–119

Communicated by K. Isono

Received January 29, 1989

J. Mol. Biol. (1989) 210, 709–719

Identification and Molecular Analysis of *oxyR*-Regulated Promoters Important for the Bacterial Adaptation to Oxidative Stress

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(Received 4 May 1989, and in revised form 5 July 1989)

The *oxyR*-encoded regulatory protein, OxyR, acts to induce the synthesis of a family of hydrogen peroxide-inducible proteins in *Salmonella typhimurium* and *Escherichia coli*. To further define the mechanism by which *oxyR* regulates the production of these proteins, we identified, mapped, and characterized *oxyR*-regulated promoters upstream from the *S. typhimurium ahp* genes (encoding an alkyl hydroperoxide reductase) and the *E. coli katG* gene (encoding catalase). A set of *ahpC* promoter deletions was constructed *in vitro* and analysis of these deletions revealed the location of sequences that are involved in *oxyR*-mediated induction of the *ahpC* gene product. DNase I protection studies of the *ahpC* promoter region revealed an *oxyR*-dependent footprint that overlapped the sequences found to be important for *oxyR* control. *E. coli* strains containing transcriptional fusions between the *katG* promoter and the *lacZ* gene showed strongly increased synthesis of β-galactosidase in response to hydrogen peroxide treatment. This stimulation was found to be *oxyR*-dependent. DNase I protection studies of the *katG* promoter region revealed an *oxyR*-dependent footprint in the same location relative to the basal promoter elements as was observed with the *ahpC* promoter. Although both the *ahpC* and *katG* promoters were shown to bind the same factor, no strong sequence similarities were found between the two, or between the two and a third *oxyR*-dependent binding site upstream from the *E. coli oxyR* gene itself.

1. Introduction

When *Salmonella typhimurium* or *Escherichia coli* cells are pretreated with low doses of hydrogen peroxide they become resistant to subsequent lethal doses of hydrogen peroxide (Demple & Halbrook, 1983; Christman *et al.*, 1985). This increased resistance is accompanied by the induction of at least 30 proteins as seen on two-dimensional gels. A gene, *oxyR*, has been identified that positively regulates the expression of at least nine of the hydrogen peroxide-inducible proteins. The *oxyR* gene has been cloned and sequenced, and the encoded protein found to show homology to a new family of regulatory proteins that includes the *Rhizobium meliloti* NodD and *E. coli* LysR proteins (Christman *et al.*, 1989). *oxyR* mutant alleles (*oxyR1* in *S. typhimurium* and *oxyR2* in *E. coli*) have been isolated that confer resistance to hydrogen peroxide and organic peroxides without pretreatment (Christman *et al.*, 1985). Cells containing such mutations also constitutively overexpress the nine *oxyR*-regulated proteins. These mutant alleles are referred to as *oxyR* constitutive mutations. The *oxyR2* constitutive mutant allele has been sequenced and shown to be a result of a single alanine to valine missense mutation (Christman *et al.*, 1989).

Some of the proteins and activities that are overexpressed in the *oxyR* constitutive mutants have been identified (Christman *et al.*, 1985). Two of the proteins (F52a and C22) comprise a novel alkyl hydroperoxide reductase activity (Jacobson *et al.*, 1989). Purification and characterization of the two proteins have shown that the F52a flavoprotein together with the C22 protein reduces lipid hydroperoxides and a variety of other organic peroxides to their corresponding alcohols. It is therefore likely that this activity plays a role in enteric bacteria analogous to that of glutathione peroxidase in mammalian cells. The *ahp* locus encoding this activity

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0022-2836/89/240709-11 $03.00/0

has been mapped to 13 minutes on the *S. typhimurium* and *E. coli* chromosomes (Storz *et al.*, 1989).

Another activity overproduced in the *oxyR* constitutive mutants is the catalase activity encoded by the *katG* gene. The *katG* gene has been mapped to 88 minutes on both the *E. coli* (Loewen *et al.*, 1985) and *S. typhimurium* (Morgan *et al.*, 1986) chromosomes. More recently, the *E. coli katG* gene has been cloned (Loewen *et al.*, 1983; Triggs-Raine & Loewen, 1987) and the DNA sequence determined (Triggs-Raine *et al.*, 1988).

In this study, we have begun to characterize the mechansims by which *oxyR* mediates the induction of the oxidative stress proteins. We report here a molecular analysis of *oxyR*-regulated promoters upstream from both the *S. typhimurium ahp* genes and the *E. coli katG* gene.

2. Experimental Procedures

(a) *Bacterial strains and plasmids*

Strains and plasmids used in this study are briefly described in Table 1. Plasmid pAQ30 was constructed by the following steps: digesting plasmid pAQ29 to completion with *Nde*I; partially digesting with *Hpa*I; filling in the *Nde*I 3′ recessed end with phage T4 DNA polymerase; ligating with phage T4 DNA ligase; and isolating clones that had lost only the DNA fragment between the *Nde*I site and the *Hpa*I site centering at position −67 relative to the start of transcription of the *ahpC* gene (as determined by restriction and nucleotide sequence analysis). Plasmid pAQ36 is also a derivative of pAQ29 and was constructed in a manner identical to that described for pAQ30, except that the enzyme used for the initial complete digestion was *Sal*I as opposed to *Nde*I. Plasmids pAQ31 through pAQ35 are deletion derivatives of plasmid pAQ36. To create the deletion derivatives, pAQ36 was linearized with *Hin*dIII and subjected to limited digestion with exonuclease *Bal*31 (New England Biolabs). This was followed by digestion with *Nde*I. The *Nde*I 3′ recessed ends were filled in with phage T4 DNA polymerase, ligated with phage T4 DNA ligase, and the DNA used to transform *oxyR2* mutant strain TA4483. The endpoints of the deletions were determined by nucleotide sequence analysis following the cloning of the deletion junctions into an M13 sequencing vector. In all constructions studied, the *Nde*I site served as the identical cloning junction, so that the various deletions could be compared. Ethidium bromide staining of mini-DNA preparations from cultures (at equal density) of both TA4482 and TA4483 carrying all deletion mutant plasmids revealed no detectable differences in plasmid copy number.

Table 1
Bacterial strains and plasmids

Designation	Description
A. *Bacterial strains*	
LT2	Wild-type *S. typhimurium*
TA4100	*oxyR1* (Christman *et al.*, 1985)
K12	Wild-type *E. coli*
TA4110	*oxyR2* (Christman *et al.*, 1985)
TA4112	*oxyRΔ3* [*oxyΔ(oxyRbtuB)3*] (*E. coli*, derived from RK4936; Christman *et al.*, 1985)
RK4936	*araD139/(argF-lac)205/glbB5301/non-9gyrA219/relA1/rpoL150/metE70/ btuB*::Tn*10* (*E. coli*)
TA4482	*ahpΔ5 zij-602*::Tn*10*
TA4483	*ahpΔ5 zij-602*::Tn*10 oxyR2*
TA4484	*oxyRΔ3*/pMC7(pMC7 carries *lacI*^{q}; Calos, 1978)
B. *Plasmids*	
pRS415	Promoter expression vector (Simons *et al.*, 1987)
pKK177-3	A derivative of pKK223-3 (Brosius & Holy, 1984) in which the *tet* gene contained on a *Bam*HI–*Pvu*II fragment has been removed
pAQ24	A 340 bp *Hin*dIII–*Eco*RI fragment from pBT22 (Triggs-Raine & Loewen, 1987) inserted into the *Sma*I polylinker site of pRS415. Results in a fusion between the *katG* promoter and the *lacZ* gene
pAQ25	*oxyR* gene cloned behind the *tac* promoter of pKK177-3. Results in overexpression of the OxyR protein upon IPTG induction (G. Storz, L. A. Tartaglia & B. N. Ames, unpublished results)
pAQ27	A 4100 base *Kpn*I–*Bgl*II fragment from pAQ9 (Storz *et al.*, 1989) containing the *ahp* locus (Fig. 1) inserted into the *Kpn*I–*Bam*HI polylinker sites of pUC18
pAQ29	A deletion derivative of pAQ27 in which sequences between the *Sma*I site and the *Sac*I site have been removed. Results in deletion of the *ahpF* gene
pAQ30	A deletion derivative of pAQ29 in which sequences between the *Nde*I site and the *Hpa*I site centering at position −67 relative to the start of transcription of the *ahpC* gene have been removed
pAQ31-pAQ35	*Bal*31 deletion derivatives of pAQ36
pAQ36	A deletion derivative of pAQ29 in which sequences between the *Sal*I site and the *Hpa*I site centering on position −67 relative to the start of transcription of the *ahpC* gene have been removed
pAQ37	A 630 bp *Hin*dIII–*Eco*RI fragment of pAQ27 cloned into the *Sma*I polylinker site of pRS415. Results in a fusion between the *ahpC* promoter and the *lacZ* gene

(b) *Immunoblots*

Samples (0·3 ml) of overnight cultures of equal density were centrifuged and the pellets resuspended in 50 μl Laemmli (1970) buffer and boiled. Portions (20 μl) were electrophoresed on 12% polyacrylamide gels. The proteins were then transferred to nitrocellulose filters by electroblotting and the filter was probed with 2 μl (1 : 5000 dilution) of C22 antibody (Storz *et al.*, 1989). Bound antibody was visualized by the reaction catalyzed by alkaline phosphatase conjugated to goat anti-rabbit antibody (Blake *et al.*, 1984; Knecht & Dimond, 1984). The nitrocellulose filters were then made transparent with microscope oil, and the intensity of the bands determined by densitometry using a Kratos SD3000 densitometer. Dilutions of extracts from TA4483 carrying pAQ30 showed the intensity of the bands to be linear with respect to the amount of protein loaded in the range studied.

(c) *Primer extension analysis*

Total cellular RNA was isolated as described by Morgan *et al.* (1986). A 30-nucleotide, end-labeled, syn-

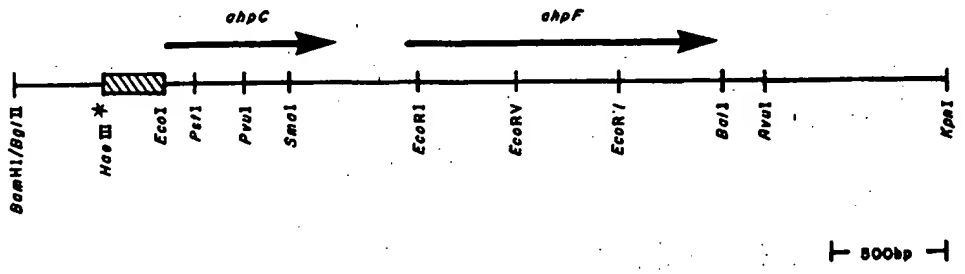

Figure 1. Restriction endonuclease map of the 4100 base *Bgl*II–*Kpn*I fragment in pAQ27 carrying the *S. typhimurium ahp* locus. The location and direction of the coding regions for the 2 components of the alkyl hydroperoxide reductase activity are indicated by arrows. The shaded region indicates the location of the *ahpC* promoter region whose sequence is shown in Fig. 3 (only 1 of several *Hae*III sites is indicated for purposes of orientation with Fig. 3).

thetic oligonucleotide was added to the indicated amounts of total cellular RNA, allowed to anneal and then extended with reverse transcriptase (Life Sciences Inc., St. Petersburg, FL: McKnight & Kingsbury, 1983). The extended products were analyzed by electrophoresis on denaturing 8% polyacrylamide gels. Dideoxy sequencing reactions primed from the same 30 base-pair end-labeled oligonucleotide were electrophoresed in neighboring lanes.

(d) *β-Galactosidase assays*

The levels of β-galactosidase in RK4936(*oxyR*$^+$) and TA4112(*oxyR*Δ*3*) carrying PAQ24 were determined as described by Miller (1982) after cells were lysed as described by Putnam & Koch (1975). The strains were grown to $A_{650} = 0{\cdot}3$, 100-μl portions were taken at 3 min intervals for 10 min before cultures were divided and half of the cultures treated with 60 μM-hydrogen peroxide. Samples were taken from both halves of the culture for an additional 30 min at 3 min intervals.

(e) *DNase I protection studies*

TA4484 carrying either pAQ25 or pKK177-3 was treated with 1 mM-isopropyl-β-D-thiogalactoside during exponential growth and then left shaking at 37 °C for an additional 2 h. Samples (5 ml) of the induced cells were centrifuged and the pellets resuspended in 800 μl of 10 mM-Tris·HCl (pH 7·5). The cells were lysed by 2 sonication steps (10 s each), centrifuged for 5 min at 3000 *g* to remove unlysed cells, followed by a 2nd centrifugation at 13,000 *g* to remove the membrane fraction. Protein concentrations of the resulting soluble fraction were determined (Bradford, 1976). For footprinting reactions (Galas & Schmitz, 1978), portions of the soluble fraction were incubated with 3 fmol of ^{32}P-end-labeled probe DNA fragment and 0·1 μg of poly(dI-dC) competitor DNA in 0·5 × TM buffer (TM buffer is 50 mM-Tris (pH 7·9), 12·5 mM-$MgCl_2$, 20% (v/v) glycerol, 1 mM-EDTA, 1 mM-dithiothreitol, 0·1% NP40, 100 mM-KCl) for 15 min at room temperature before 25 μl of 10 mM-$MgCl_2$, 5 mM-CaCl and 2 μl of 1 to 3 μg DNase I/ml (Cooper Biomedical) were added and the incubation continued for 1 min. The reaction was stopped by the addition of 200 μl of stop buffer (20 mM-EDTA (pH 8·0), 1% (w/v) SDS, 0·2 M-NaCl, 250 μg RNA/ml), extracted with phenol and chloroform, precipitated, and analyzed on a denaturing 8% polyacrylamide gel in parallel with a G/A sequencing ladder (Galas & Schmitz, 1978).

3. Results

(a) *Identification of an* oxyR-*regulated promoter upstream from the* S. typhimurium ahp *locus*

In a previous study, we reported the isolation of a clone from a *S. typhimurium* library that complemented the peroxide sensitivity of *ahp* deletion mutants and restored the expression of the C22 and F52a proteins that comprise the alkyl hydroperoxide reductase activity (Storz *et al.*, 1989). We have subcloned and sequenced the *ahp* locus contained on this clone and verified that it contains the two structural genes encoding both the C22 and F52a proteins (L. A. Tartaglia, G. Storz, M. Brodsky & B. N. Ames, unpublished results). The two genes were found to be closely linked and transcribed in the same direction, with the *ahpC* gene (encoding the C22 protein) located upstream from the *ahpF* gene (encoding the F52a protein: see Fig. 1). The translation start points of both genes were identified by comparison of the translated open reading frames with the N-terminal amino acid sequences of the purified C22 and F52a proteins (Jacobson *et al.*, 1989).

The transcription initiation site upstream from the *ahpC* gene was determined by primer extension studies using reverse transcriptase. A 30 bp† primer was hybridized to total cellular RNA isolated from LT2 (wild-type *S. typhimurium*) and extended along the *ahpC* mRNA toward its 5′ end. The extended primer was then electrophoresed alongside dideoxy sequencing reactions primed with the same 30 bp primer (Fig. 2). One major band resulted from the extension of the primer, and by comparison with the adjacent sequencing ladder the transcription start point was identified. The adenine residue corresponding to the transcription start point is indicated as position +1 on Figure 3, which shows 222 bp of sequence surrounding the start of the *ahpC* gene. Inspection of sequences upstream from the transcription start revealed −10 and −35 regions resembling the sigma 70 consensus sequence.

† Abbreviation used: bp, base-pair(s).

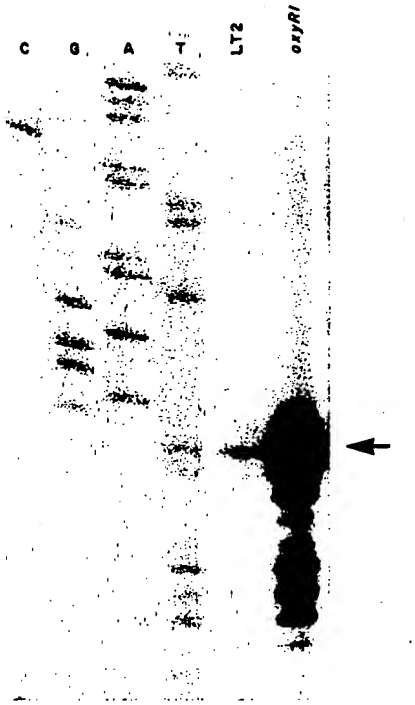

Figure 2. Primer extension analysis of the *ahpC* transcript. A 30 bp oligonucleotide was hybridized with 30 μg of RNA from strain LT2 or 30 μg of RNA from strain *oxyR1* (TA4100) and the primer extended using reverse transcriptase. The resulting fragments were analyzed on an 8% polyacrylamide gel together with dideoxy sequencing ladders that were primed with the same oligonucleotide as used for the primer extension analysis. The major band indicating the start of the *ahpC* transcript from both LT2 and *oxyR1* is indicated by an arrow on the right.

In order to determine if this promoter was regulated by the *oxyR* locus, an identical experiment was simultaneously performed on an equal amount of total cellular RNA isolated from an *oxyR1* mutant strain (TA4100). The *oxyR1* strain has been shown to constitutively overproduce (treatment with hydrogen peroxide is not required) the nine *oxyR*-regulated proteins, including the *ahpC* gene product (Christman *et al.*, 1985). Primer extension of *oxyR1* RNA showed the same major band as was observed in the LT2 RNA primer extension. However, a comparison of the extended products showed a significant increase in the steady-state level of *ahpC* mRNA in the constitutive mutant background. This result suggested that the basal level promoter identified by the LT2 RNA primer extension can be strongly stimulated by the *oxyR* locus.

(b) *Deletion analysis of the* ahpC *promoter*

To further characterize the *oxyR* stimulation of the *ahp* promoter, we examined the regulation of the cloned promoter in both wild-type and constitutive *oxyR* mutant backgrounds. Constitutive *oxyR* mutant strains were not viable when transformed with plasmids containing the *ahp* promoter either in front of the two *ahp* genes or in transcriptional fusions with the *lacZ* gene. This may have been due to the inability of cells to tolerate the high levels of these enzymes produced when the corresponding genes are present in multiple copies and are stimulated by the constitutive *oxyR* mutant allele. However, constitutive *oxyR* mutant strains transformed with plasmids containing only the *ahp* promoter and the *ahpC* gene (the *ahpF* gene deleted) were found to be viable. Using such constructs, we were then able to detect *oxyR* stimulation of the *ahpC* gene by immunologically quantifying the relative levels of the C22 protein produced in wild-type and constitutive *oxyR* mutant backgrounds (see below). So that comparisons could be made with the *E. coli katG* promoter (see below). these regulatory studies were done in an *E. coli* $oxyR^+$ strain and an *E. coli* strain containing the constitutive *oxyR2* mutation, which is analogous to the *S. typhimurium oxyR* mutant allele *oxyR1* (Christman *et al.*, 1985).

Plasmid pAQ30, which contains the *ahpC* gene and 67 nucleotides of upstream non-transcribed sequence (see Fig. 3), was transformed into $oxyR^+$ *ahpΔ5* (TA4482) and *oxyR2 ahpΔ5* (TA4483). The C22 protein produced from pAQ30 could be visualized clearly in extracts of both strain backgrounds when analyzed by SDS/polyacrylamide gel electrophoresis and stained with Coomassie blue (data not shown). Immunological quantification of the relative levels of C22 protein produced in the two strain backgrounds showed a sixfold induction by the constitutive *oxyR* mutant allele (see Table 2). This C22 protein induction is in good agreement with the fivefold induction of the chromosomally encoded

Table 2
Induction of the ahpC *gene product by the constitutive* oxyR2 *mutant allele*

Plasmid	Extent of deletion	Fold-induction
pAQ30	−67	6·1 ± 0·5 (n = 4)
pAQ31	−59	2·9 ± 0·3 (n = 3)
pAQ32	−46	1·1 ± 0·3 (n = 3)
pAQ33	−41	0·9 ± 0·2 (n = 3)
pAQ34	−34	1·0 ± 0·3 (n = 3)
pAQ35	−20	—

ahpC promoter deletion plasmids pAQ30 through pAQ35 were transformed into TA4482 ($oxyR^+$ *ahpΔ5*) and TA4483 (*oxyR2 ahpΔ5*). The resulting 12 strains were then grown to stationary phase and the whole cell extracts analyzed by immunoblot analysis with anti-C22 antibody (see Experimental Procedures). The fold-induction indicated for each plasmid reflects the ratio of the C22 protein produced in the *oxyR2* constitutive mutant strain carrying that plasmid over that produced in the $oxyR^-$ strain carrying the same plasmid. The standard deviation of the fold-induction and number of trials (n) is indicated. The extent of the deletion into the *ahpC* promoter relative to the transcription start point is shown in the middle column.

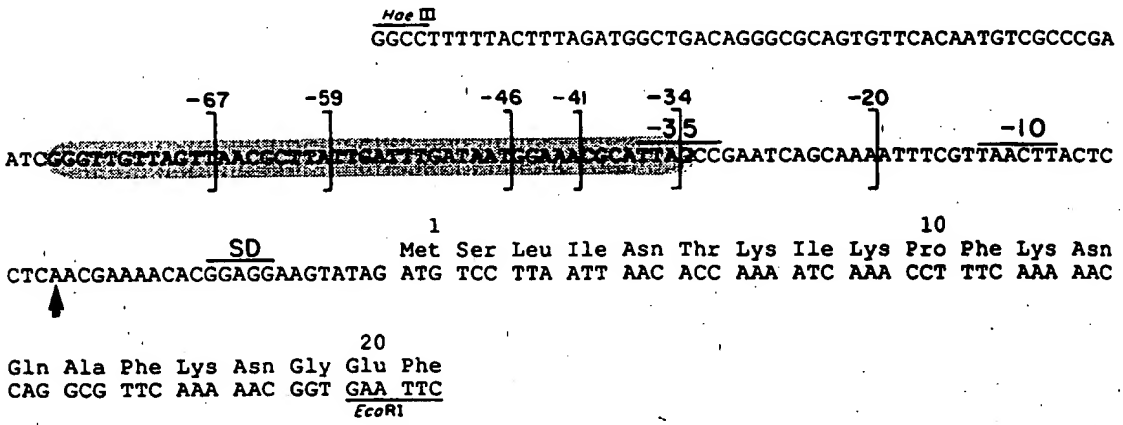

Figure 3. Nucleotide sequence of a *Hae*III–*Eco*RI fragment of pAQ27 containing the *ahpC* promoter region. The mRNA start point deduced from primer extension analysis is indicated by a bold arrow. Putative Shine–Dalgarno (SD) and sigma 70 −10 and −35 regions are marked by a line drawn above the relevant nucleotides. The start of translation of the *ahpC* message is indicated by the translation of the relevant codons into the corresponding N-terminal amino acid residues of the C22 protein (L. A. Tartaglia, G. Storz, M. Brodsky & B. N. Ames, unpublished results). The end points of the *Hpa*I and *Bal*31-generated deletions, as determined by sequence analysis, are identified by brackets with the deletion position relative to the transcription start indicated. The position of the OxyR-dependent binding site, as deduced by DNase I protection analysis, is indicated by the shaded oval.

alkyl hydroperoxide reductase activity observed in *oxyR1* strains under similar conditions (Christman *et al.*, 1985). This demonstrates that the *S. typhimurium ahpC* gene is expressed and regulated by *oxyR* in *E. coli*, and that sequences sufficient for *oxyR* stimulation are located downsteam from position −68. Primer extension analysis of mRNA isolated from both *oxyR*$^+$ *ahpΔ5* and *oxyR2 ahpΔ5* containing pAQ30 showed that the induction was also reflected in the steady-state level of *ahpC* mRNA and that the transcript initiation site of the cloned gene in *E. coli* was identical with that observed originating from the *S. typhimurium* chromosome (data not shown). No *ahp* transcript was detected by this assay in the *E. coli ahpΔ5* controls that did not contain pAQ30.

To determine if sequences upstream from *ahpC* mediate its regulation by *oxyR*, a set of 5′ deletions was generated downstream from position −67 using *Bal*31 nuclease (see Experimental Procedures). Five deletion mutant plasmids (pAQ31–pAQ35) were chosen for detailed analysis and transformed into the *oxyR*$^+$ *ahpΔ5* strain TA4482 and the *oxyR2 ahpΔ5* strain TA4483. The extent of the deletions was determined by nucleotide sequence analysis and is shown in Figure 3. A comparison of the relative amount of C22 protein produced from each deletion mutant plasmid in the *oxyR*$^+$ and *oxyR2* mutant backgrounds is shown in Table 2. Quantification of the relative amount of C22 protein produced by strains *oxyR*$^-$ *ahpΔ5* and *oxyR2 ahpΔ5* carrying pAQ31 revealed a threefold stimulation by the *oxyR2* mutant allele. Deletion plasmids pAQ32, pAQ33 and pAQ34 all produced the C22 protein at easily detectable levels in both strain backgrounds; however, no stimulation by the constitutive *oxyR* allele was observed, indicating that sequences required for *oxyR* stimulation were lost. No C22 protein was detected under the conditions of our assay in extracts of either *oxyR*$^+$*ahpΔ5* or *oxyR2-ahpΔ5* carrying pAQ35, suggesting that sequences required for basal level expression were removed in this deletion mutant. The above studies indicate that sequences between −67 and −46 contain at least a portion of the *cis*-acting element involved in the stimulation of the *ahpC* promoter by *oxyR*.

(c) *Extracts from strains that overproduce the OxyR protein protect sequences upstream from* ahpC *from DNase I digestion*

To determine whether the OxyR protein or OxyR-mediated factors interact with the sequences upstream from *ahpC* shown to be important in OxyR control by the deletion analysis, we prepared extracts from otherwise isogenic *E. coli* strains that either produced no OxyR protein (TA4484 containing pKK177-3) or that overproduced the *E. coli* OxyR protein (TA4484 containing pAQ25). These extracts were then assayed for a protein factor that could specifically bind sequences upstream from *ahpC*. To assay for binding, we monitored the protection from limited DNase I digestion. Figure 4 (lanes 4 and 5) shows that extracts from the strain that overproduces the OxyR protein specifically protect sequences upstream from *ahpC*. The footprint extends from approximately position −79 to the −35 region. Lanes 2 and 3 show the results of an identical

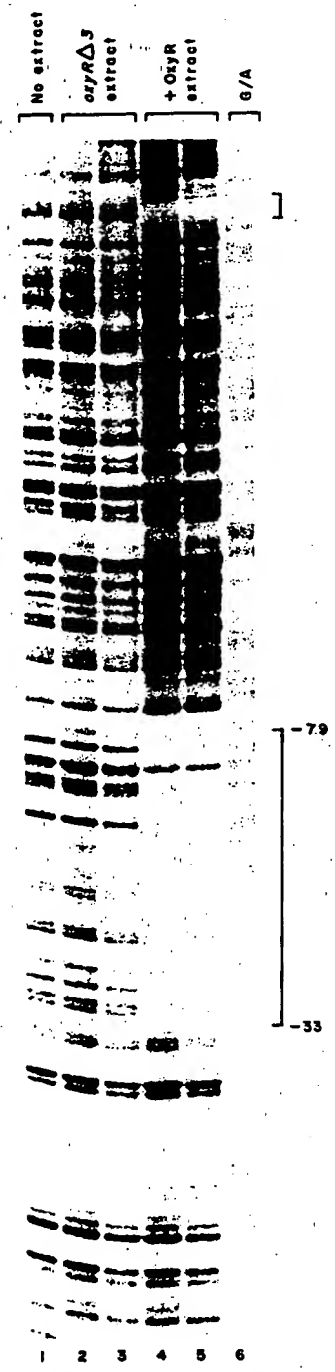

Figure 4. DNase I protection analysis of the *ahpC* promoter. A 630 bp *Hin*dIII–*Eco*RI fragment of pAQ27 was ^{32}P-labeled at the *Eco*RI 5′ end. The labeled fragment was incubated with either extract buffer alone (lane 1), extract prepared from *oxyR* deletion strain TA4484 carrying control plasmid pKK177-3 at final total protein concentrations of 4·6 μg/ml (lane 2) and 46 μg/ml (lane 3), or extract prepared from *oxyR* deletion strain TA4484 carrying OxyR-overproducing plasmid pAQ25 at final total protein concentrations of 4·6 μg/ml (lane 4) and 46 μg/ml (lane 5). After DNase I digestion, samples were run in parallel with a Maxam–Gilbert G/A sequencing ladder (lane 6) on an 8% polyacrylamide sequencing gel. The regions protected from DNase I digestion are indicated by brackets at the right.

experiment done with extracts that do not contain the OxyR protein, and demonstrate that the protection is OxyR-dependent. The protected sequences between positions −79 and −33 (see Fig. 3) are in good agreement with the results of the deletion analysis, which indicate the presence of *cis*-acting *oxyR* regulatory sequences between −67 and −46. Lanes 4 and 5 in Figure 4 show evidence for another OxyR-dependent DNase I-protected region, approximately 300 bp upstream from the *ahpC* promoter. The function of this upstream binding site is not understood, since *ahpC* clones containing over 600 bp of upstream sequence do not show significantly greater induction of the *ahpC* gene product than that observed for pAQ30, which contains only 67 nucleotides of upstream non-transcribed sequence (data not shown).

(d) *Identification of a hydrogen peroxide- and* oxyR-*regulated promoter upstream from the* E. coli katG *gene*

Constitutive *oxyR1* and *oxyR2* mutants have significantly higher levels of catalase activity than wild-type strains (Christman *et al.*, 1985). These increased catalase levels have been attributed to an increase in the steady-state levels of *katG* mRNA (Morgan *et al.*, 1986). To determine whether sequences upstream from the *E. coli katG* gene could bestow hydrogen peroxide and *oxyR* regulation upon a promoterless *lacZ* gene, a 340 bp *Hin*dIII–*Eco*RI fragment from pBT22 (Triggs-Raine & Loewen, 1987) containing the start of the *katG* structural gene and 141 bp of upstream sequence was cloned into the promoterless *lacZ* expression vector pRS415. The fragment was cloned in an orientation such that promoters upstream from the *katG* gene should transcribe the *lacZ* gene of pRS415. This construct (pAQ25) was then transformed into both an *oxyR*$^+$ strain (RK4936) and an otherwise isogenic *oxyR* deletion strain (TA4112). Due to the high basal expression of the *katG* promoter and the transient nature of hydrogen peroxide induction, we assayed induction of the *katG* promoter by using the method for assaying transient induction phenomenon described by Taylor *et al.* (1984). Levels of β-galactosidase in cultures of the wild-type and *oxyR* deletion strains carrying the transcriptional fusion were determined during mid-log growth. The cells were then divided and half the cultures treated with 60 μM-hydrogen peroxide. Each culture was monitored for an additional 30 minutes for levels of β-galactosidase and cell density. Figure 5 shows the levels of β-galactosidase as reflected by the conversion of *o*-nitrophenyl-β-D-

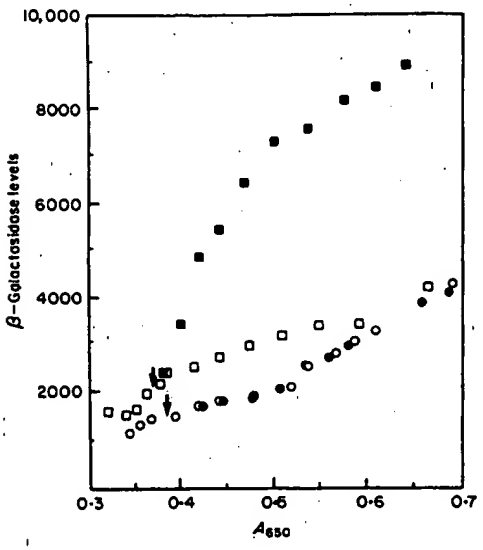

Figure 5. Induction of β-galactosidase in strains carrying *katG-lacZ* fusion plasmids by treatment with hydrogen peroxide. Cultures of *oxyR*$^+$ (RK4936) and *oxyRΔ3* (TA4112) strains carrying a chimeric *katG* promoter-*lacZ* gene construct (pAQ24) were divided and half of each of the cultures brought to a final concentration of 60 μM-hydrogen peroxide. The times of hydrogen peroxide treatment are indicated by vertical arrows. Portions of the cultures were taken every 3 min, lysed, and assayed for β-galactosidase activity. The levels of β-galactosidase (defined as $1000 \times [A_{420} - 1{\cdot}75 \times A_{550}] / [t(\text{min}) \times v(\text{ml})]$) are plotted *versus* A_{650}. (□) *oxyR*$^+$; (■) *oxyR*$^+$ with hydrogen peroxide; (○) *oxyRΔ3*; (●) *oxyRΔ3* with hydrogen peroxide.

Figure 6. Primer extension analysis of the *katG* transcript. A 30 bp oligonucleotide was hybridized with 35 μg of RNA from strain K12 or 35 μg of RNA from strain *oxyR2* (TA4110) and the primer extended using reverse transcriptase. The resulting fragments were analyzed on an 8% polyacrylamide gel together with dideoxy sequencing ladders that were primed with the same oligonucleotide as used for the primer extension analysis. The major band indicating the start of the *katG* transcript from both K12 and *oxyR2* is indicated by an arrow.

galactoside to *o*-nitrophenyl plotted *versus* cell density. The sharp increase in slope (indicating an increased rate of β-galactosidase synthesis) seen for the wild-type treated but not the wild-type untreated cells demonstrates that the 340 bp *katG* fragment can confer hydrogen peroxide-inducibility upon the *lacZ* gene. Treatment with hydrogen peroxide did not affect the β-galactosidase synthesis in an *oxyR* deletion strain, indicating that the induction is *oxyR*-dependent. The slightly higher levels of β-galactosidase observed in untreated wild-type cells compared to *oxyR* deletion cells was consistently observed in several experiments. This effect may be due to a small amount of *oxyR*-dependent induction of *katG* during normal aerobic growth. We have constructed a transcriptional fusion between the *lacZ* gene of pRS415 and the promoter of the *ahpC* gene (pAQ37, described in Table 1). Induction levels and kinetics for the *ahpC* fusion were very similar to those seen with the *katG* fusion (data not shown).

The transcription initiation site upstream from the *katG* gene was determined by primer extension analysis. A 30 bp primer was hybridized to total cellular RNA isolated from K12 and *oxyR2* strains, and extended along the *katG* mRNA towards its 5′ end. The extended primers were then electrophoresed alongside dideoxy sequencing reaction mixtures primed with the same 30 bp primer (Fig. 6). From the adjacent sequencing ladder, the transcription startpoint was identified as an adenine residue marked +1 in Figure 7, and is identical for both the basal level transcription and the highly induced level of transcription observed in the constitutive *oxyR2* mutant. Inspection of sequences upstream from the transcription start revealed −10 and −35 regions resembling the sigma 70 consensus sequence that are in agreement with those predicted by Triggs-Raine *et al.* (1988) on the basis of sequence homology.

(e) *DNase I protection studies of the* E. coli katG *gene*

DNase I protection experiments similar to those described for the *ahpC* promoter (identical extracts) were performed with a DNA fragment containing sequences upstream from the *katG* gene (Fig. 8).

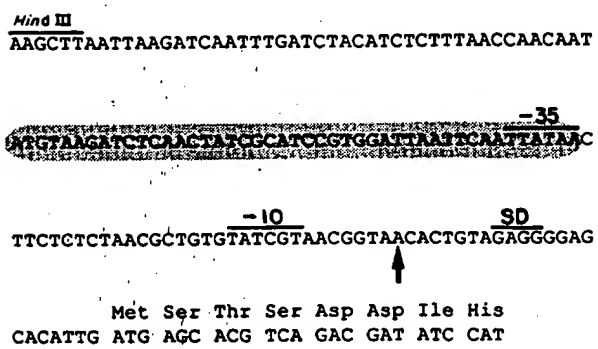

Figure 7. Nucleotide sequence of the *katG* promoter region. The transcription start point deduced from primer extension analysis is marked by a bold arrow. Putative Shine–Dalgarno (SD) and sigma 70 −10 and −35 regions are indicated by a line drawn above the relevant nucleotides. The start of translation of the *katG* message is indicated by the translation of the relevant codons into the corresponding N-terminal amino acid residues of the *katG* gene product (Triggs-Raine *et al.*, 1988). The position of the OxyR-dependent binding site as deduced by DNase I protection analysis is indicated by the shaded oval.

These studies revealed an OxyR-dependent footprint upstream from the *katG* promoter starting at position −76 and extending through the −35 consensus sequence (see Fig. 7). The position of this footprint relative to the promoter elements is nearly identical with that observed for the *ahpC* gene. Interestingly, however, visual inspection of the two protected regions showed no obvious sequence similarities. Computer alignment of the two protected regions using the SEARCH algorithm in the Intelligenetics SEQ program (IG Suite version 5.2, Intelligenetics, Palo Alto, CA) also revealed no homologies significantly better than those expected if the two sequences were randomized. The best homology (expect value = 0·4 occurrences on average) was a 14 bp stretch in which 10 bp were conserved. However, we feel it is unlikely that this match has physiological significance, since the region is centrally located in the *ahpC* footprint, while proximally located in the *katG* footprint, and no similar sequence is found in a third OxyR-dependent binding site (see below). Also, computer analysis of the DNase I-protected regions upstream from both *ahpC* and *katG* revealed no dyad symmetries better than would be expected in random sequences of this size.

(f) *The* ahpC *and* katG *promoters compete for the binding of the same factor*

The dissimilarity between the two OxyR-dependent binding sites upstream from the *ahpC* and *katG* promoters raised the question of whether the two promoter fragments were binding the same factor. Possibly two different isoforms of the OxyR protein recognize the two different sequences, or expression

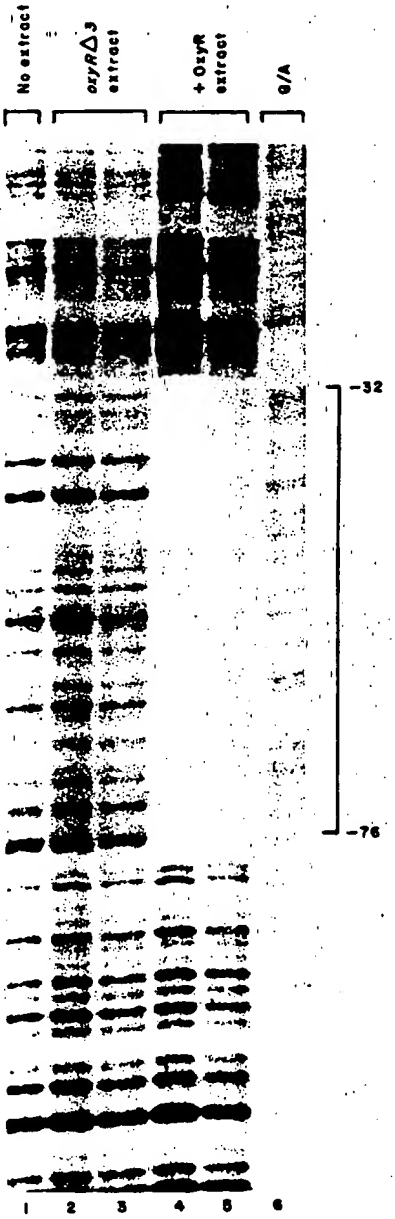

Figure 8. DNase I protection analysis of the *katG* promoter. A 194 bp *Eco*RI–*Eco*RV fragment of pBT22 was ^{32}P-labeled at the *Eco*RI 5′ end. The labeled fragment was incubated with either extract buffer alone (lane 1), extract prepared from *oxyR* deletion strain TA4484 carrying control plasmid pKK177-3 at final total protein concentrations of 4·6 μg/ml (lane 2) and 23 μg/ml (lane 3), or extracts prepared from *oxyR* deletion strain TA4484 carrying OxyR-overproducing plasmid pAQ25 at final total protein concentrations of 4·6 μg/ml (lane 4) and 23 μg/ml (lane 5). After DNase I digestion, samples were run in parallel with a Maxam–Gilbert G/A sequencing ladder (lane 6) on an 8% polyacrylamide sequencing gel. The regions protected from DNase I digestion are indicated by brackets at the right.

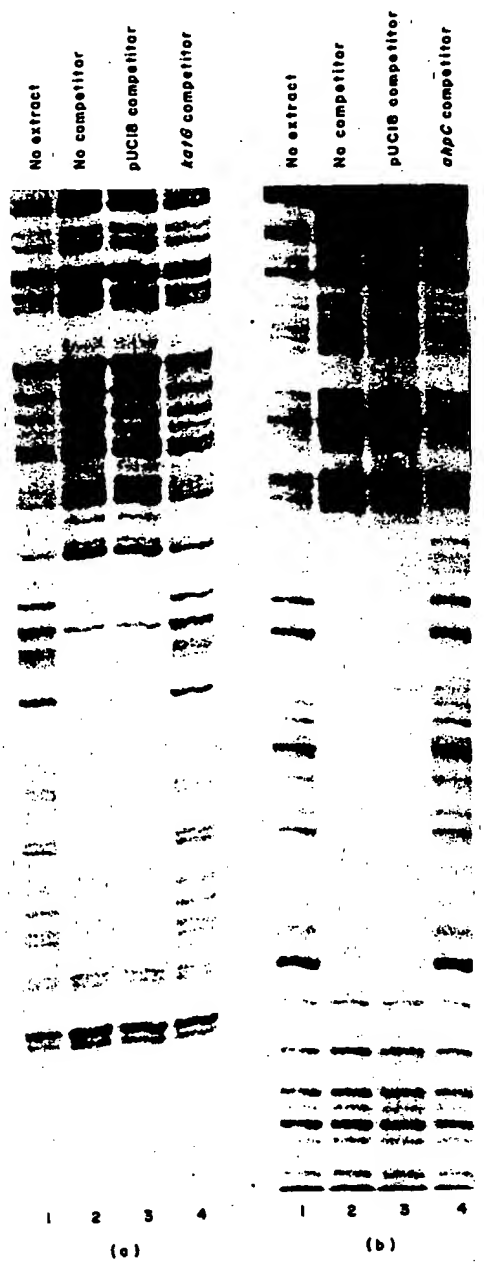

Figure 9. Competition study with the *katG* and *ahpC* promoters. (a) A 630 bp *Hin*dIII–*Eco*RI fragment of pAQ27 was ^{32}P-labeled at the *Eco*RI 5′ end. The labeled fragment was incubated with either extract buffer alone (lane 1), or extract prepared from *oxyR* deletion strain TA4484 carrying OxyR-overproducing plasmid pAQ25 at a final total protein concentration of 4·6 μg/ml (lanes 2 through 4). A 55-fold molar excess of unlabeled competitor DNA fragments was also added to some lanes as follows: lanes 1 and 2, no competitor DNA fragment; lane 3, 55-fold molar excess (relative to *ahpC* probe fragment) of *Nde*I–*Sal*I fragment from pUC18; lane 4, 55-fold molar excess of *Eco*RI–*Eco*RV fragment of pBT22 containing the *katG* promoter. After DNase I digestion, samples were run on an 8% polyacrylamide sequencing gel. (b) A 194 bp *Eco*RI–*Eco*RV fragment of pBT22 was ^{32}P-labeled at the *Eco*RI 5′ end. The labeled fragment was incubated with either extract buffer alone (lane 1), or extract prepared from *oxyR* deletion strain TA4484 carrying OxyR-overproducing plasmid pAQ25 at a final total protein concentration of 4·6 μg/ml (lanes 2 through 4). A 55-fold molar excess of unlabeled competitor DNA fragment was also added to some lanes as follows: lanes 1 and 2, no competitor DNA fragment; lane 3, 55-fold molar excess (relative to *katG* probe fragment) of *Nde*I–*Sal*I fragment from pUC18; lane 4, 55-fold molar excess of *Hin*dIII–*Eco*RI fragment of pAQ27 containing *ahpC* promoter. After the DNase I digestion, the samples were run on an 8% polyacrylamide sequencing gel.

of OxyR results in the binding of other factors to the two promoter sequences. To help distinguish between these possibilities, we performed competition experiments with the two promoter fragments. Figure 9(a) shows that the OxyR-dependent footprint on the *ahpC* promoter could be competed with a 55-fold molar excess of a *katG* promoter fragment containing a single OxyR-dependent binding site (the more upstream footprint of unknown sequence on the *ahp* fragment was also competed, data not shown). The *ahpC* footprint is not competed by a 55-fold molar excess of a 230 bp pUC18 DNA fragment. Therefore, despite the dissimilarity in sequence of the two protected regions, the factor binding upstream from *ahpC* appears to be the same as that binding upstream from *katG*. The reciprocal experiment is shown in Figure 9(b). We have recently found an OxyR-dependent footprint just upstream from the *oxyR* gene itself (Christman *et al.*, 1989). The sequence of the protected region upstream from the *oxyR* gene is shown in Figure 10. Competition experiments similar to those described above have shown that a 55-fold molar excess of either the *ahpC* or *katG* promoter fragments could compete for the binding of the OxyR-dependent factor binding upstream from the *oxyR* gene (data not shown). A 55-fold molar excess of a pUC18 fragment did not compete. Interestingly, no statistically significant similarities were found between the OxyR binding site upstream from *oxyR* and the OxyR binding sites upstream from either the *katG* or *ahpC* gene in either orientation.

4. Discussion

Promoters upstream from both the *S. typhimurium ahpC* gene and the *E. coli katG* gene were identified and localized. The steady-state levels of mRNA produced from these two promoters were found to be substantially higher in *oxyR* constitutive mutant strains. This, together with the finding that altering non-transcribed sequences upstream from *ahpC* eliminates *oxyR* stimulation, suggests that *oxyR* regulates at the level of transcription initiation.

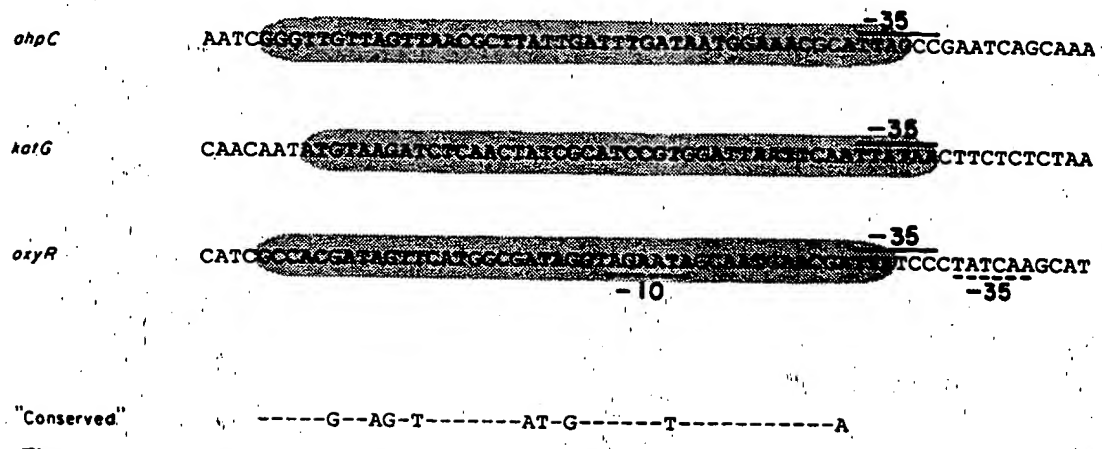

Figure 10. Optimal alignment of three OxyR-dependent binding sites. The shaded ovals indicate the sequences upstream from the *ahpC*, *katG* and *oxyR* genes that are protected by OxyR-enriched extracts in DNase I protection assays. The −35 regions indicated on the *ahpC* and *katG* nucleotide sequences correspond to those described above. The −35 region indicated by the bold line on the *oxyR* nucleotide sequence corresponds to that of a positively regulated small transcript made opposite the *oxyR* gene (G. Storz & B. N. Ames, unpublished results). The broken lines indicate the positions of the −10 and −35 regions of the *oxyR* gene itself (Christman *et al.*, 1989). Nucleotides that are the same for all 3 sequences in this alignment are shown on the bottom line.

E. coli soluble extracts enriched for the OxyR protein were shown to protect sequences upstream from the *ahpC* and *katG* promoters. No protection was seen in experiments performed with extracts from otherwise isogenic strains that did not produce the OxyR protein. The protected sequences upstream from *ahpC* include a region defined by deletion mapping as a *cis*-acting *oxyR* regulatory element. Although it is still possible that the OxyR protein mediates the protection upstream from the *ahpC* and *katG* promoters by activating another binding factor, we do not favor this hypothesis, because substantially purified OxyR preparations show the same protection patterns (G. Storz, L. A. Tartaglia & B. N. Ames, unpublished results).

The location of the OxyR footprint on the *katG* and *ahpC* promoters is also of interest. Both of the OxyR footprints extend into the −35 sigma 70 consensus sequence, which typically also makes contacts with RNA polymerase (Siebenlist *et al.*, 1980; von Hippel *et al.*, 1984). It has been shown that both the bacteriophage lambda cII protein and RNA polymerase interact with the −35 region of several lambda promoters by making contacts at this region on opposite sides of the DNA helix (Ho *et al.*, 1983). This observation has led to the proposal that cII activation may be a result of direct protein–protein interaction between the cII protein and RNA polymerase. The protection patterns upstream from the *ahpC* and *katG* promoters are therefore consistent with a model in which the OxyR protein stimulates these promoters by making contacts with RNA polymerase.

The extracts used for the DNase I protection experiments in this study were prepared from cultures that had not been treated with hydrogen peroxide. The results obtained therefore suggest that the OxyR protein can bind DNA with reasonable affinity and specificity even in the absence of hydrogen peroxide treatment. However, it is possible also that the OxyR binding activity was activated by oxidation upon release from the reducing environment of an *E. coli* cell. In fact, preliminary *in vitro* transcription studies indicate that the transcription stimulatory activity of OxyR becomes activated simply upon extraction into air-saturated buffers. We have found, however, that the factor still binds these promoters under strongly reducing conditions (G. Storz, L. A. Tartaglia & B. N. Ames, unpublished results). These same reducing conditions have been found to inhibit the ability of these extracts to stimulate transcription of the *ahp* locus *in vitro* (L. A. Tartaglia, G. Storz & B. N. Ames, unpublished results). Before any comparisons can be made between the relative binding affinity of this factor in its active and inactive forms, further studies will be required to determine the exact nature of the activation process and the conditions under which the activation process takes place.

There are many examples in prokaryotes of sets of genes that are co-ordinately controlled by the binding of a common regulatory factor. These co-ordinately regulated genes typically contain a consensus sequence in their 5′ flanking DNA that is recognized by the regulatory factor. It was therefore surprising that comparisons of the DNA sequence contained in three OxyR-dependent footprints showed no statistically significant similarities. This observation is even more unexpected in light of the finding that these three sequences appear to bind

the same factor, which is most likely the OxyR protein itself. The binding of this regulatory factor to the *ahpC*, *katG* and *oxyR* 5′-regions is therefore atypical of the pattern normally seen with other bacterial regulatory proteins. However, the binding of a common regulatory protein to apparently non-homologous *cis*-regulatory elements has been reported in a number of eukaryotic systems. One of these is the yeast HAP1 protein, which binds sequences upstream from both the CYC1 and CYC7 genes (Pfeifer *et al.*, 1987). Like the OxyR binding sites, no obvious homologies were found between these two binding sites in either orientation. Other examples include the COUP transcription factor, which binds sequences upstream from both the chicken ovalbumin gene and the rat insulin II gene (Hwung *et al.*, 1988), and the HeLa cell TEF-1 protein, which binds simian virus 40 (SV40) enhancer motifs of unrelated sequence (Davidson *et al.*, 1988). Some of these investigators have speculated that DNA secondary structure may be important in the binding specificity of these factors (Pfeifer *et al.*, 1987; Hwung *et al.*, 1988). This principle may be operating in the case of the OxyR-regulated promoters; however, not enough DNA structural information is available to allow speculation about secondary structures present at these or most other protein-binding sites.

It is possible also that the recognition of these sites is still governed by primary sequence information, since there are alignments of the three binding sites in which several positions are conserved. The optimal and probably most physiologically significant alignment is shown in Figure 10. However, the distance over which positions of identity are scattered and the uncharacteristic absence of dyad symmetries makes it difficult to distinguish important similarities from similarities expected between random sequences. Recently, Bölker & Kahmann have found that the OxyR protein also binds upstream from the *mom* gene in the phage Mu (Bölker & Kahmann, 1989). Although it is not known if the *mom* binding site will efficiently compete for the binding of OxyR at the *ahp*, *katG* and *oxyR* promoters, we have attempted to align all four binding sites. Several possible alignments exist in which either five or six residues out of the nine-residue consensus proposed in Figure 10 are conserved. To determine which of these alignments is most significant and to understand how the OxyR protein recognizes its binding sites will require further protection, interference and mutant binding studies.

We thank Steve Bell for assistance with the footprinting experiments and Peter Loewen for sending us the *katG* clone. This work was supported by National Institutes of Health (NIH) grant GM19993 and National Cancer Institute Outstanding Investigator grant CA39910 to B.N.A. and by National Institute of Environmental Health Sciences (NIEHS) Center grant ES01896. L.A.T. was supported by NIEHS Training grant ES07075 and G.S. was supported by NIH Training grant GM07232.

References

Blake, M. S., Johnston, K. H., Russell-Jones, G. J. & Gotschlich, E. C. (1984). *Anal. Biochem.* **136**, 175–179.

Bölker, M. & Kahmann, R. (1989). *EMBO J.* **8**, 2403–2410.

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.

Brosius, J. & Holy, A. (1984). *Proc. Nat. Acad. Sci., U.S.A.* **81**, 6929–6933.

Calos, M. P. (1978). *Nature (London)*, **274**, 762–765.

Christman, M. F., Morgan, R. W., Jacobson, F. S. & Ames, B. N. (1985). *Cell*, **41**, 753–762.

Christman, M. F., Storz, G. & Ames, B. N. (1989). *Proc. Nat. Acad. Sci., U.S.A.* **86**, 3484–3488.

Davidson, I., Xiao, J. H., Rosales, R., Staub, A. & Chambon, P. (1988). *Cell*, **54**, 931–942.

Demple, B. & Halbrook, J. (1983). *Nature (London)*, **304**, 466–468.

Galas, D. J. & Schmitz, A. (1978). *Nucl. Acids Res.* **5**, 3157–3170.

Ho, Y.-S., Wulff, D. L. & Rosenberg, M. (1983). *Nature (London)*. **304**, 703–708.

Hwung, Y.-P., Crowe, D. T., Wang, L.-H., Tsai, S. Y. & Tsai, M.-J. (1988). *Mol. Cell. Biol.* **8**, 2070–2077.

Jacobson, F. S., Morgan, R. W., Christman, M. F. & Ames, B. N. (1989). *J. Biol. Chem.* **264**, 1488–1496.

Knecht, D. A. & Dimond, R. L. (1984). *Anal. Biochem.* **136**, 180–184.

Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.

Loewen, P. C., Triggs, B. L. & Klassen, G. R. (1983). *Canad. J. Biochem. Cell Biol.* **61**, 1315–1321.

Loewen, P. C., Triggs, B. L., George, C. S. & Hrabarchuk, B. E. (1985). *J. Bacteriol.* **162**, 661–667.

McKnight, S. L. & Kingsbury, R. (1983). *Science*, **217**, 316–324.

Miller, J. H. (1982). Editor of *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Morgan, R. W., Christman, M. F., Jacobson, F. S., Storz, G. & Ames, B. N. (1986). *Proc. Nat. Acad. Sci., U.S.A.* **83**, 8059–8063.

Pfeifer, K., Prezant, T. & Guarente, L. (1987). *Cell*, **49**, 19–27.

Putnam, S. L. & Koch, A. L. (1975). *Anal. Biochem.* **63**, 350–360.

Siebenlist, U., Simpson, R. B. & Gilbert, W. (1980). *Cell*, **20**, 269–281.

Simons, R. W., Houman, F. & Kleckner, N. (1987). *Gene*, **53**, 85–96.

Storz, G., Jacobson, F. S., Tartaglia, L. A., Morgan, R. W., Silveira, L. A. & Ames, B. N. (1989). *J. Bacteriol.* **171**, 2049–2055.

Taylor, W. E., Straus, D. B., Grossman, A. D., Burton, Z. F., Gross, C. A. & Burgess, R. R. (1984). *Cell*, **38**, 371–381.

Triggs-Raine, B. L. & Loewen, P. C. (1987). *Gene*, **52**, 121–128.

Triggs-Raine, B. L., Doble, B. W., Mulvey, M. R., Sorby, P. A. & Loewen, P. C. (1988). *J. Bacteriol.* **170**, 4415–4419.

von Hippel, P. H., Bear, D. G., Morgan, W. D. & McSwiggen, J. A. (1984). *Annu. Rev. Biochem.* **53**, 389–446.

Edited by M. Gottesman

JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 330–338
0021-9193/97/$04.00+0

Vol. 179, No. 2

Identification and Characterization of Hydrogen Peroxide-Sensitive Mutants of *Escherichia coli*: Genes That Require OxyR for Expression

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Received 4 June 1996/Accepted 1 November 1996

Escherichia coli **produces an inducible set of proteins that protect the cell from exogenous peroxide stress. A subset of these genes is induced by hydrogen peroxide and is controlled at the transcriptional level by the OxyR protein. To identify additional genes involved in protection from hydrogen peroxide, a library of random transcriptional fusions of λ*plac*Mu53 was screened for hydrogen peroxide sensitivity and 27 such mutants were identified. These fusions were transduced into nonlysogenic strains to ensure that the phenotypes observed were the result of a single mutation. The mutants were grouped into three classes based on the expression of the *lacZ* fusion during growth in *oxyR*$^+$ and Δ*oxyR* backgrounds. The expression of the *lacZ* fusion in 8 mutants was independent of OxyR, 10 mutants required OxyR for expression, and 6 mutants showed reduced levels of expression in the presence of OxyR. OxyR dependence varied from 2- to 50-fold in these mutants. The OxyR-dependent phenotype was complemented by a plasmid-borne copy of *oxyR* gene in all mutants. Three mutants exhibited dual regulation by OxyR and RpoS. We sequenced the fusion junctions of several of these mutants and identified the genetic loci responsible for the hydrogen peroxide-sensitive (*hps*) phenotype. In this study, we report the identification of several genes that require OxyR for expression, including *hemF* (encoding coproporphyrinogen III oxidase), *rcsC* (encoding a sensor-regulator protein of capsular polysaccharide synthesis genes), and an open reading frame, *f497*, that is similar to arylsulfatase-encoding genes.**

All aerobic respiring organisms require protection from reactive oxygen species (including superoxide anion, hydroxyl radical, and hydrogen peroxide) formed from the partial reduction of molecular oxygen to water during oxidative metabolism. Bacterial cells encounter endogenous hydrogen peroxide produced from the dismutation of superoxide or hydroxyl radical as a product of the respiratory chain when oxygen is used as the terminal electron acceptor. In addition, enteric bacteria, such as *Salmonella typhimurium* and *Escherichia coli*, encounter toxic levels of hydrogen peroxide produced by macrophages during engulfment (17). *E. coli* and *S. typhimurium* possess several enzymes that prevent oxidative damage (alkyl hydroperoxidase, catalases, superoxide dismutase, and glutathione reductase) and repair DNA lesions resulting from oxidative damage (e.g., exonuclease III, RecBC nuclease, and endonuclease III) (for a review, see reference 16). It has been shown that both *E. coli* (14) and *S. typhimurium* (49) become resistant to killing by hydrogen peroxide when pretreated with a nonlethal dose (60 μM) of hydrogen peroxide. The adaptation results in the transient accumulation of a distinct group of proteins (10, 28). There are 30 proteins that exhibit elevated level of synthesis, of which 12 proteins are induced immediately after the hydrogen peroxide challenge and 18 proteins are expressed 10 to 30 min after hydrogen peroxide treatment (10). The induction of 9 of the 30 proteins is under positive control of the *oxyR* gene product (10, 28), OxyR, a member of the LysR family of transcriptional regulators (11). Genes known to be induced by OxyR in the presence of sublethal doses of hydrogen peroxide include *katG* (encoding HPI catalase) (10, 28, 39), *ahpCF* (encoding alkyl hydroperoxidase reductase) (10, 28, 39), *gorA* (encoding glutathione reductase) (21), *dps* (a nonspecific DNA-binding protein with a protective function) (3), and *oxyS* (encoding a small untranslated RNA with a probable regulatory function) (21, 22). OxyR protein also represses its own synthesis (10, 39, 42) as well as that of the Mu phage *mom* gene (6). Other members of the OxyR regul n which are overexpressed in a constitutive *oxyR* mutant are yet to be identified. An OxyR-independent hydrogen peroxide-inducible gene product has been identified as DnaK protein (encoded by *dnaK* gene), which is involved in DNA biosynthesis (28). Some proteins induced under hydrogen peroxide stress are also induced by other forms of stress, including those generated by heat, ethanol, nalidixic acid, and cumene peroxide (28).

To identify members of the hydrogen peroxide (oxidative stress)-induced regulon that are responsible for protecting the cell from oxidative damage, a previously reported (36) rand m library of transposon-generated *lacZ* operon fusions in *E. coli* (λ*plac*Mu53 fusion phage) was screened for sensitivity to hydrogen peroxide and 26 mutants that showed a hydrogen peroxide-sensitive (*hps*) phenotype toward 1.0 mM hydrogen peroxide (on plate assays) were isolated. Here we present the characterization of the *hps* genes that require OxyR for their expression. The identities of these OxyR-activated *hps* genes are reported on the basis of the DNA sequence of the fusion junctions that map to known regions of the *E. coli* chromosome, thereby identifying some of the genes responsible for producing an Hps phenotype.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. All bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1.

Chemicals and enzymes. All chemicals were obtained from commercial sources. Stock solutions of antibiotics and other nonautoclavable solutions were filter sterilized (pore size, 0.45 μm; Gelman Sciences, Ann Arbor, Mich.) prior to use.

Media used and growth conditions. The rich medium used was Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl) adjusted to pH 7.0. For preparation and determination of the titers of P1*vir* lysates, cultures were grown overnight at 37°C with 5 mM $CaCl_2$. The P1-mediated transductions were performed by the method of Miller (27). Solid medium was prepared by

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TABLE 1. *E. coli* strains, phages, and plasmids used in this study

Strain(s), phage, or plasmid	Genotype	Source and/or reference
Strains		
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA deoC1 ptsF25 rbsR flb5301*	G. Weinstock
GC4468	F⁻ Δ*lacU169 rpsL*	Laboratory collection
KL765	F⁻ *lacZ813 lac13 pro met his trp rpsL thi* λ*ind*	K. B. Low
HS008	Same as KL765 but with ϕ80	Laboratory collection
HS6682	Same as HS008 but *uvrD*::Tn*5*	Laboratory collection
RK4936	*araD139* Δ(*argF-lac*)*205 flb5301* non-*gyrA219 relA1 rpsL150 btuB*::Tn*10*	10
TA4112	Same as RK4936 but *oxyΔ3*[*oxy*(*oxyR-btuB*)*3*]	10
TA4484	Same as TA4112 but has pMC7 (*lacI*^{q})	21
GC202	Same as GC4468 but *katG17*::Tn*10*	Laboratory collection
GC122	Same as GC4468 but *rpoS13*::Tn*10*	Laboratory collection
NC202	Same as GC4468 but Φ(*katE*::*lacZ*⁺)*131 katG17*::Tn*10*	Laboratory collection
NC4468	Same as GC4468 but Φ(*katE*::*lacZ*⁺)*131*	Laboratory collection
HS701 to HS727	Same as MC4100 but Φ(*hps-1*::*lacZ*⁺) to Φ(*hps-27*::*lacZ*⁺)	This study
HS701R to HS727R	Same as HS701 to HS727 but *rpoS13*::Tn*10*	P1(GC122) × HS701 to HS727→Tet^r
GC701 to GC727	Same as GC4468 but Φ(*hps-1*::*lacZ*⁺) to Φ(*hps-27*::*lacZ*⁺)	This study
RK701 to RK727	Same as RK4936 but Φ(*hps-1*::*lacZ*⁺) to Φ(*hps-27*::*lacZ*⁺)	This study
TA701 to TA727	Same as TA4112 but Φ(*hps-1*::*lacZ*⁺) to Φ(*hps-27*::*lacZ*⁺)	This study
Phages		
λp*lac*Mu53	λ*imm 'trp' 'lacZ*⁺ *lacY*⁺ *lacA' 'uvrD' Xho*::*kan* cIts62 *ner*⁺ *A*⁺*'S*	G. Weinstock
P1*vir*		Laboratory collection
Plasmids		
pAQ24	*katG* promoter fused to a promoterless *lacZ*	G. Storz (43)
pAQ25	*oxyR* gene cloned in pKK177-3 with *tac* promoter	G. Storz (21)
pMC7	Plasmid containing *lacI*^{q} gene	G. Storz (21)

adding 15 g of agar per liter of liquid medium. The soft top agar was prepared by adding 7.5 g of agar per liter of liquid medium. For titration of λ phage, the host strain was grown with 10 mM $MgSO_4$ and 0.2% maltose.

Single-colony isolates were inoculated into growth medium supplemented with appropriate antibiotics and incubated overnight at 37°C. All cultures were grown in flasks at a culture/flask volume ratio of 1/5 to ensure good aeration. Growth was monitored by measuring optical density at 600 nm (OD_{600}) with a Shimadzu UV1201 UV-VIS spectrophotometer. For all induction experiments, overnight cultures were serially subcultured twice to an OD_{600} of 0.2 before inoculation into fresh media to an initial OD_{600} of 0.05 to 0.10 or as indicated.

Identification of hydrogen peroxide-sensitive mutant strains. A collection of more than 5,000 random *lacZ* operon fusions to chromosomal promoters were isolated by infecting strain MC4100 with λp*lac*Mu53 (transcriptional fusion phage) as described previously (36, 37). Cultures of individual colonies were grown in 96-well microtiter plates, replica plated onto LB plates and LB plates containing 1.0 mM hydrogen peroxide, and grown overnight at 37°C. Colonies that exhibited poor growth in the presence of hydrogen peroxide were presumptive hydrogen peroxide-sensitive mutants. These presumptive mutants were further tested for catalase activity by flooding the replicates with 30% hydrogen peroxide. Eight hydrogen peroxide-sensitive mutants also exhibited reduced bubbling in the presence of hydrogen peroxide and thus had reduced catalase activity. P1*vir* lysates were prepared (27) on the hydrogen peroxide-sensitive mutants, and the *lacZ* fusion was transduced into strains GC4468 and MC4100 to ensure that only one fusion was present in each mutant derivative. These transductants were tested for hydrogen peroxide sensitivity. The same P1*vir* lysates were also used to transduce the fusions into strains RK4936 (*oxyR*⁺) and TA4112 (Δ*oxyR*).

Cell survival assays. Six single-colony isolates from each bacterial strain were inoculated into a 96-well microtiter plate containing 0.1 ml of LB per well and incubated at 37°C until saturation. The cultures were replica plated (in duplicate) onto LB agar and LB agar containing 1.0 mM hydrogen peroxide and incubated overnight at 37°C. Relative sensitivity was determined by comparing the growth of mutant strains with those of appropriate parental strains.

To quantitate the survival of cultures exposed to hydrogen peroxide, overnight cultures were serially subcultured twice to an OD_{600} of 0.20 (approximately 3 × 10^8 cells/ml). The second subculture of each strain was divided into two portions. One portion of the culture was challenged with 1.0 mM hydrogen peroxide (final concentration), while the other served as a control. The cultures were incubated for 60 min at 37°C and 200 rpm. Aliquots of cultures were withdrawn at the indicated times, appropriately diluted, plated onto LB plates in duplicate, and incubated overnight at 37°C. The relative survival was assessed by determining the viable count of treated cultures in relation to that of untreated cultures incubated in parallel. A concentration of 1 mM hydrogen peroxide was used to challenge the growing cultures because this concentration causes "mode-one" killing in *E. coli* that is dependent on cellular metabolism (19). For determining the zone of inhibition, cultures were grown to an OD_{600} of 0.8 and a 200-μl aliquot was plated onto an LB plate with 3 ml of soft agar. The overlaid plates were allowed to dry at room temperature for 30 min. A Whatman (number 3) filter disc (diameter, 7 mm) soaked with 10 μl of 30% hydrogen peroxide was placed on the middle of each overlaid plate and incubated at 37°C, and the diameter of the zone of inhibition of bacterial growth was measured after 12 h.

Enzymatic assays. β-Galactosidase assays were performed by the method of Miller (27). Activities were normalized with respect to culture density and were expressed in Miller units (27).

Plasmid isolation and transformation. Plasmids were isolated from overnight cultures grown in media containing appropriate antibiotics by the alkaline lysis method (35). Competent cells were prepared by the calcium chloride method (35) and were transformed with a total of 50 ng of either plasmid pAQ25 or pMC7 (21, 22). Transformants were selected on LB plates containing 100 μg of ampicillin per ml (pAQ25) or 15 μg of tetracycline per ml (pMC7). For double transformation, 250 ng of total DNA (125 ng of each plasmid) was used. The double transformants were selected on LB plates containing both ampicillin and tetracycline. Six individual isolates from each transformed mutant strain were inoculated into 96-well microtiter plates along with parent strains and appropriate controls and grown at 37°C to saturation. Microtiter wells were replica plated onto plates supplemented with 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml with or without 1 mM isopropyl-thio-D-galactosidase (IPTG) as inducer. The plates were incubated at 37°C for 12 h, and the change in color was examined in the presence and absence of IPTG.

Induction of cultures with IPTG. Overnight cultures were serially subcultured twice in LB containing appropriate antibiotics to an initial OD_{600} of 0.2. Cultures were divided into two equal portions; to one portion, IPTG was added to a final concentration of 1 mM. Cultures were incubated at 37°C at 200 rpm. Aliquots of cultures were removed every 30 min, placed on ice with chloramphenicol (150 μg/ml [final concentration]) to stop further protein synthesis, and assayed for β-galactosidase activity. All assays were performed in duplicate.

Induction of λp*lac*Mu53 lysogens by UV and DNA isolation from lysates. To isolate phage DNA, λp*lac*Mu53 lysogens were induced by UV as described previously (33, 37). An overnight culture from a single-colony isolate was subcultured to an OD_{600} of 0.4 (approximately 3.4 × 10^8 cells/ml of culture) in 50 ml of LB. The culture was centrifuged at 7,000 × *g* at 4°C for 15 min, resuspended in 10 ml of 10 mM $MgSO_4$, and spread on a petri dish (15-cm diameter). The culture suspension was irradiated uncovered for 6 s by placing it 50 cm below two 15-W germicidal UV lamps (NIS G15T8). Total fluence was approximately 31 J/m^2, as measured with a UVX radiometer (Ultraviolet Light Products, San Gabriel, Calif.) and a 254-nm probe (model no. UVX-25). To the irradiated cell

suspension, 5.0 ml of 3× LL broth (90 g of tryptone, 45 g of yeast extract, 45 g of NaCl, 60 mg [each] of adenine, cytosine, guanine, and thymine per liter, pH 7.25) was added and incubated at 37°C with vigorous shaking (200 rpm) until visible lysis occurred (3 to 5 h). The lysate was transferred to 50-ml polypropylene screwcap tubes (catalog no. 25330-50; Corning Inc., Corning, N.Y.) containing a few drops of chloroform and vortexed vigorously for 1 min before being centrifuged at 3,000 rpm for 20 min at 4°C to remove cell debris. An aliquot of the lysate was appropriately diluted and plated with strain MC4100 as host onto LB plates containing 50 μg (each) of streptomycin and X-Gal per ml (phage titers varied from 10^9 to 10^7 PFU/ml). The phage was pelleted by centrifuging the lysate at 35,000 rpm for 30 min in a Beckman SW41Ti rotor (33). DNA from the pelleted phage was isolated by a previously described method (35).

DNA sequencing of the fusion junctions and analysis. The mutator phage employed in this study, λp*lac*Mu53 (7), is a hybrid phage containing λ and phage Mu segments. To identify chromosomal genes immediately adjacent to the integrated phage, we used a DNA sequencing primer complementary to the Mu C end. The primer, AB3818, was a 25-mer, 5′ CCGGAATAATCCAATGTCCTC CCGG 3′, 30 nucleotides downstream from the Mu C end (33). The phage DNA isolated from UV-induced lysates was purified by using a glass membrane ultrafiltration cartridge (catalog no. 15590-060; Gibco BRL, Burlington, Ontario, Canada) as recommended by the manufacturer. The amount of DNA used in each sequencing reaction was 10 to 100 fmol. Sequencing of the phage templates was performed with *Taq* polymerase (AmpliCycle sequencing kit; Perkin-Elmer, Branchburg, N.J.) by using a 5′-end-labelled primer, AB3818, with [α-^{35}S]thio-dATP (>1,000 Ci/mmol). The sequencing reaction was performed in 25 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 60 s with a thermal cycler (GeneE thermal cycler; Techne Inc., Princeton, N.J.) equipped with a heated lid (33). The sequences of the fusion junctions were determined from two independent sequencing reactions performed each time with freshly prepared templates. DNA sequences were examined for homology with the data available in the GenBank database by using the program (2).

RESULTS

Isolation of hydrogen peroxide-sensitive mutants containing chromosomal *lacZ* operon fusions. Transcriptional fusion phage λp*lac*Mu53 (7) was used to obtain a random chromosomal *lacZ* operon fusion bank of over 5,000 operon fusions, as described in Materials and Methods. On screening the fusion bank, 27 mutant strains (in the MC4100 background) which were sensitive to 1.0 mM hydrogen peroxide were isolated. Subsequently, the *lacZ* fusions were transduced into strains GC4468 and MC4100 to ensure that only one fusion was present in each mutant derivative. The genes corresponding to the fusions were designated *hps* (hydrogen peroxide-sensitive phenotype). As shown in Table 2, each of these fusions was sensitive to 1.0 mM hydrogen peroxide and exhibited a larger zone of inhibition than did the wild-type strain (refer to Materials and Methods).

Quantitative cell survival assays were performed on most of the *hps* mutants (data not reported). We report the sensitivities of a set of representative mutants (*hps-2*, *hps-4*, *hps-5*, and *hps-10*) compared to those of mutant *hps-7* in isogenic *oxyR*$^+$ and Δ*oxyR* backgrounds (Fig. 1). Exponentially growing cultures were challenged with 1 mM hydrogen peroxide to determine the effect on viability, as *E. coli* undergoes mode-one killing when exposed to 1 to 2 mM hydrogen peroxide (19). At this concentration of hydrogen peroxide, additional cellular responses (apart from the known OxyR-dependent, SOS-mediated, or superoxide-inducible response) that are as yet uncharacterized are induced (19). All the *hps* mutant strains were sensitive (3- to 10-fold) to 1.0 mM hydrogen peroxide. The *hps*Δ*oxyR* double mutants were more sensitive than were the *hps* mutant strains (data not reported). The *hps-7*Δ*oxyR* double mutant exhibited a synergistic hypersensitive phenotype and had an approximately 50-fold-reduced viability compared to that of the wild-type strain, indicating that other genes (such as *hps-7*) may be involved in hydrogen peroxide stress response independent of OxyR.

Effect of OxyR on β-galactosidase expression of *hps*::*lacZ* fusions. Hydrogen peroxide treatment induces the synthesis of 30 proteins in *S. typhimurium* (28). The synthesis of nine f these proteins, including HPI hydroperoxidase, is regulated by OxyR (10, 28), a transcriptional activator (39, 42). Since we isolated 27 *hps* mutants, it was possible that some of the fusions were in OxyR-regulated genes. To determine the effect f OxyR on the expression of various *hps*::*lacZ* mutants, the fusions were transduced into isogenic *oxyR*$^+$ (strain RK4936) and Δ*oxyR* (strain TA4112) backgrounds. However, we were unable to obtain viable transductants for fusion *hps-17*::*lacZ* in an Δ*oxyR* background. All of the transductants were sensitive to hydrogen peroxide in an *oxyR*$^+$ background compared to the parent strain (Table 2), as determined by the radius of inhibition.

As an initial screen, overnight cultures of *hps*::*lacZ* fusions in *oxyR*$^+$ and Δ*oxyR* backgrounds were assayed for β-galactosidase activity to identify OxyR-dependent fusions (Table 3). The mutants could be separated into the following three major classes: OxyR independent (the *oxyR*$^+$/Δ*oxyR* ratio, ≅1); OxyR

TABLE 2. Sensitivities of *hps* mutants to hydrogen peroxide[a]

Strain	Allele	Sensitivity to hydrogen peroxide (mM)[b]		Radius of inhibition (mm)[c]	
		0.0	1.0	*oxyR*$^+$	Δ*oxyR*
RK4936	Wild type	+	+	7.5	15.5[e]
GC4468	Wild type	+	+	9.0	
NC4468	*katE*::*lacZ*	+	±	9.2	
GC202	*katG*::Tn*10*	+	−	11.5	
NC202	*katE*::*lacZ*, *katG*::Tn*10*	+	−	19.5	
HS701	*hps-1*	+	−	10.3	17.3
HS702	*hps-2*	+	−	10.8	22.2
HS703	*hps-3*	+	−	9.5	20.8
HS704	*hps-4*	+	−	12.5	18.7
HS705	*hps-5*	+	−	10.8	20.2
HS706	*hps-6*	+	−	9.0	18.8
HS707	*hps-7*	+	−	11.3	24.0
HS710	*hps-10*	+	−	12.2	21.2
HS711	*hps-11*	+	−	12.5	15.8
HS712	*hps-12*	+	−	8.8	18.8
HS713	*hps-13*	+	−	11.3	17.5
HS714	*hps-14*	+	−	8.8	17.8
HS715	*hps-15*	+	−	11.5	21.8
HS716	*hps-16*	+	−	9.3	19.0
HS717	*hps-17*	+	−	9.0	NA[d]
HS718	*hps-18*	+	−	8.7	20.2
HS719	*hps-19*	+	−	8.8	19.7
HS720	*hps-20*	+	−	8.8	17.0
HS721	*hps-21*	+	−	10.5	17.2
HS722	*hps-22*	+	−	8.7	16.8
HS723	*hps-23*	+	−	11.7	18.2
HS724	*hps-24*	+	−	9.0	21.2
HS725	*hps-25*	+	−	9.7	19.3
HS726	*hps-26*	+	−	9.3	19.3
HS727	*hps-27*	+	−	8.8	16.8

[a] Single-colony isolates were inoculated into 96-well microtiter plates, grown at 37°C to saturation, and replica plated onto LB plates and LB plates containing 1.0 mM hydrogen peroxide, as described in Materials and Methods. In *hps-8* and *hps-9* mutants, the fusion junction was in the *katE* gene; hence, they were not included in this table or subsequent studies.

[b] +, growth; ±, slight growth; −, no growth.

[c] A 0.2-ml aliquot of a culture grown for 6 h was plated on an LB plate with 3 ml of LB soft agar. A filter disc (diameter, 7 mm) with 10 μl of 30% hydrogen peroxide was placed in the middle of each overlaid petri dish and incubated for 12 h at 37°C. Data are averages of triplicate plate assays. The standard error for each set of experiments was less than 0.2.

[d] NA, not available. The *hps-17*::*lacZ* fusion could not be transduced into an Δ*oxyR* background.

[e] Radius of inhibition for isogenic Δ*oxyR* strain TA4112.

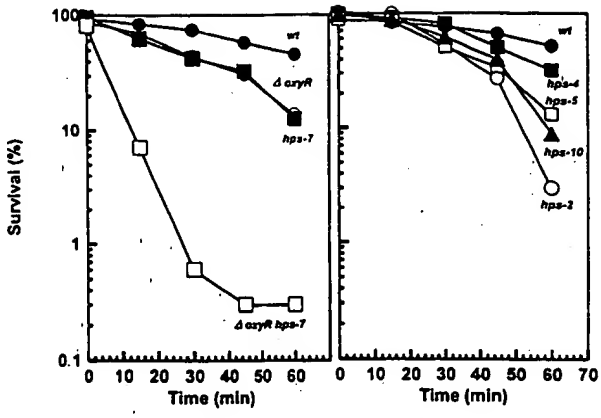

FIG. 1. Effect of 1 mM hydrogen peroxide on survival of *hps* mutants. Exponentially growing cultures were challenged with 1 mM hydrogen peroxide and assayed for survival over a period of 60 min, as described in Materials and Methods. The wild-type (*wt*) strain was RK4936, and the Δ*oxyR* strain was TA4112. The mutant strains exposed to the challenge were HS702 (*hps-2*::*lacZ*), HS704 (*hps-4*::*lacZ*), HS705 (*hps-5*::*lacZ*), HS707 (*hps-7*::*lacZ*), and HS710 (*hps-10*::*lacZ*).

activated, showing a decreased level of β-galactosidase expression of an *hps*::*lacZ* fusion in an *oxyR* deletion background (*oxyR*$^+$/Δ*oxyR* ratio, >2); and OxyR repressed, showing a higher level of *lacZ* expression in an *oxyR* deletion background (*oxyR*$^+$/Δ*oxyR* ratio, <0.5).

Effect of OxyR on growth phase-dependent expression of *hps*::*lacZ* fusions. OxyR can act as a transcriptional activator (39, 42, 43) and a repressor, repressing its own expression (10) and that of the *mom* gene of phage Mu (6). Since the role of OxyR has been studied using exponential-phase cultures (10, 28, 29) and the differences in expression of the *hps*::*lacZ* fusions in overnight cultures were modest between *oxyR*$^+$ and Δ*oxyR* backgrounds for most fusions (Table 3), we decided to study the expression of the *hps*::*lacZ* fusions in isogenic *oxyR*$^+$ and Δ*oxyR* backgrounds during normal growth to determine which of these *hps* loci are activated or repressed by OxyR. Each of these *hps*::*lacZ* operon fusions showed individual variable expression patterns during growth, indicating that each fusion is unique in its regulation. Depending on the pattern of *lacZ* expression, the fusions were grouped into one of three classes (Table 4). The *hps*::*lacZ* fusions whose levels were independent of the status of OxyR were assigned to class I (*hps-1*, *hps-3*, *hps-7*, *hps-11*, *hps-12*, *hps-15*, *hps-18*, and *hps-23*) (Fig. 2A). Ten mutants were grouped as class II fusions because they were OxyR activated (required functional OxyR for maximal expression of the *lacZ* fusion; *hps-2*, *hps-4*, *hps-5*, *hps-10*, *hps-14*, *hps-16*, *hps-20*, *hps-21*, *hps-24*, and *hps-27*). A representative member of this group, *hps-21*::*lacZ*, showed growth phase-dependent expression in an *oxyR*$^+$ background and had almost 60-fold-higher activity than that of an isogenic Δ*oxyR* strain (Fig. 2B). All other members of this group exhibited 5- to 50-fold-higher expression of the fusion in an *oxyR*$^+$ background than in an Δ*oxyR* background. Unlike in class II mutants, in class III mutants the expression of the *lacZ* fusion was repressed by a functional OxyR protein. During exponential phase of growth, the expression of these fusions was 2- to 10-fold lower in the isogenic *oxyR*$^+$ background compared to that in the Δ*oxyR* background (data not shown). The maximum difference in expression was significant enough to classify these fusions as OxyR-repressed genes (*hps-6*, *hps-13*, *hps-19*, *hps-22*, *hps-25*, and *hps-26*). A representative member of this class of mutant is *hps-19* (Fig. 2B). The class III mutants will be discussed in a subsequent communication.

TABLE 3. Effect of OxyR on the expression of *hps* fusions[a]

Fusion	β-Galactosidase activity (Miller units)		*oxyR*$^+$/Δ*oxyR* ratio
	oxyR$^+$	Δ*oxyR*	
hps-1::*lacZ*	16.7	40.4	0.4
hps-2::*lacZ*	92.0	67.0	1.4
hps-3::*lacZ*	89.3	88.3	1.0
hps-4::*lacZ*	67.3	12.0	5.6
hps-5::*lacZ*	18.8	14.1	1.3
hps-6::*lacZ*	8.0	15.2	0.5
hps-7::*lacZ*	1.1	1.2	0.9
hps-10::*lacZ*	97.0	80.1	1.2
hps-11::*lacZ*	89.3	88.9	1.0
hps-12::*lacZ*	18.7	10.7	1.7
hps-13::*lacZ*	1.2	43.7	0.03
hps-14::*lacZ*	30.1	18.5	1.6
hps-15::*lacZ*	36.8	37.3	1.0
hps-16::*lacZ*	53.1	15.1	3.6
hps-18::*lacZ*	95.0	83.4	1.1
hps-19::*lacZ*	6.7	43.4	0.2
hps-20::*lacZ*	63.0	13.0	4.8
hps-21::*lacZ*	65.8	4.7	14.0
hps-22::*lacZ*	21.3	51.7	0.4
hps-23::*lacZ*	17.0	23.2	0.7
hps-24::*lacZ*	54.0	17.5	3.1
hps-25::*lacZ*	60.2	84.5	0.7
hps-26::*lacZ*	79.3	96.5	0.8
hps-27::*lacZ*	60.9	12.6	4.8

[a] Overnight cultures grown in LB were assayed for β-galactosidase activity.

Complementation of *hps* mutants by a plasmid-borne *oxyR* gene. As some of the *hps*::*lacZ* mutants exhibited OxyR-dependent expression, a plasmid-borne copy of the *oxyR* gene (21) was used to confirm the phenotype by complementing the expression of the *hps*::*lacZ* fusion in an Δ*oxyR* background. All *hps* mutants in the TA4112 background (Δ*oxyR*) were transformed with plasmids pAQ25, containing an IPTG-inducible *oxyR* construct (p*tac-oxyR*), and pMC7 (*lacI*^{q}) (21). The double transformants were replica plated onto X-Gal-containing plates with and without 1 mM IPTG (as described in Materials and Methods) to confirm complementation. Subsequently, expression assays were performed with cultures as described in Materials and Methods. The results (Table 5) indicate that all class II mutants were complemented by the *oxyR* gene and the degree of complementation varied from 2- to 14-fold. Previ us efforts to complement these mutants with a plasmid construct of the *oxyR* gene containing wild-type OxyR resulted in modest levels of complementation, probably due to the autoregulatory effect of OxyR (data not shown). All class III mutants were also complemented by OxyR with respect to the repression phenotype (data not shown).

Induction of *hps*::*lacZ* fusions with 60 μM hydrogen peroxide. Hydrogen peroxide induces the synthesis of 30 proteins in *S. typhimurium*, of which 9 are regulated by OxyR (10, 28). Since the mutations in *hps* genes conferred a hydrogen peroxide-sensitive phenotype and since some of the *hps*::*lacZ* fusions appeared to be activated by OxyR, we tested whether these *hps*::*lacZ* fusions were hydrogen peroxide inducible (i.e., exhibited increased expression in presence of 60 μM hydrogen peroxide) in accordance with the known function of OxyR protein. Seven OxyR-activated fusions exhibited some degree of inducibility in the presence of 60 μM hydrogen peroxide (Table 6). A plasmid construct carrying a *katG* promoter fused

TABLE 4. Classification of *hps::lacZ* mutants

Class	Characteristic(s)	Fusions	Known mutants with similar phenotypes[a]
I	Independent of OxyR	*hps-1, hps-3, hps-7, hps-11, hps-12, hps-15, hps-18, hps-23*	*sodA, sodB, xthA, dnaK*
II	OxyR activated; requirement of functional OxyR for maximum expression	*hsp-2, hps-4, hps-5, hps-10, hps-14, hps-16, hps-20, hps-21, hps-24, hps-27*	*katG, ahpCF, gorA, dps, oxyS*
III	OxyR repressed; expression of the fusion reduced in the presence of OxyR	*hps-6, hps-13, hps-19, hps-22, hps-25, hps-26*	*oxyR, mom* (ΦMu)

[a] See text for discussion and references.

to a promoterless *lacZ* gene (43) was transformed into an *oxyR*$^+$ strain (RK4936). Its induction was used to measure OxyR activation and served as a control. There was 1.5- to 5-fold induction of various *hps::lacZ* fusions in the presence of 60 μM hydrogen peroxide (Table 6). The *hps-16::lacZ* fusion exhibited fivefold induction, while *hps-21::lacZ* exhibited modest twofold induction.

Regulation of *hps* loci by RpoS. The expression of various *hps::lacZ* fusions during entry into stationary phase (Fig. 2) suggests that these loci are regulated by RpoS, a stationary-phase-dependent sigma factor (24, 26, 41). The β-galactosidase activities in these mutants increased sharply as cultures entered the stationary phase of growth, as shown in the case of *hps-21::lacZ* (Fig. 3). Since it is known that the expression of *katG* (23, 29), *dps* (3), and *gorA* (4) genes is regulated by both OxyR (in a hydrogen peroxide-inducible manner) and RpoS in a growth phase-dependent manner, we wanted to determine whether any of the *hps* genes were also regulated by RpoS. An *rpoS*::Tn*10* allele was transduced from strain GC122 into all the *hps* mutants in the MC4100 background. The transductants were screened for reduced β-galactosidase activities by replica plating onto X-Gal-containing plates, as described in Materials and Methods. Five *rpoS hps* double mutants exhibited lower β-galactosidase activities on X-Gal plates than did the isogenic *rpoS*$^+$ strain. The activity levels of the *hps::lacZ* fusions in these strains were determined during normal growth, and three of these *hps* genes showed various degrees (2- to 10-fold) of dependence on RpoS (*hps-5*, *hps-19*, and *hps-21*) (Table 7). The *hps-21::lacZ* mutant showed more-than-threefold dependence on RpoS, similar to those of some previously known genes, such as *katG* (23, 29), *gorA* (4), and *dps* (3) (Fig. 3).

Identification of some of the *lacZ* fusion junctions by DNA sequencing. To identify *hps* genes mutated by the insertion of λp*lac*Mu53 phage, the integrated phage was induced from cultures and sequenced, as described in Materials and Methods. Here we report the fusion junctions of class II *hps* mutants (OxyR activated) that mapped to previously known genes of *E. coli*, thereby identifying new members of the OxyR regul n (Table 8). The fusion junction in *hps-27* is in a gene encoding a sensor protein of a two-component regulatory pathway of colanic acid capsular polysaccharide synthesis, RcsC (40). The insertion is in a proline codon 671 bases from the initiation of translation (40). The fusion junctions in *hps-5* (*thiG*) (5, 47), *hps-10* (*gldA*) (5, 46), and *hps-24* (*uvrD*) (13, 50) are oriented opposite to the known promoter transcribing the gene. In *hps-24::lacZ*, the insertion is in the noncoding strand, 497 bases

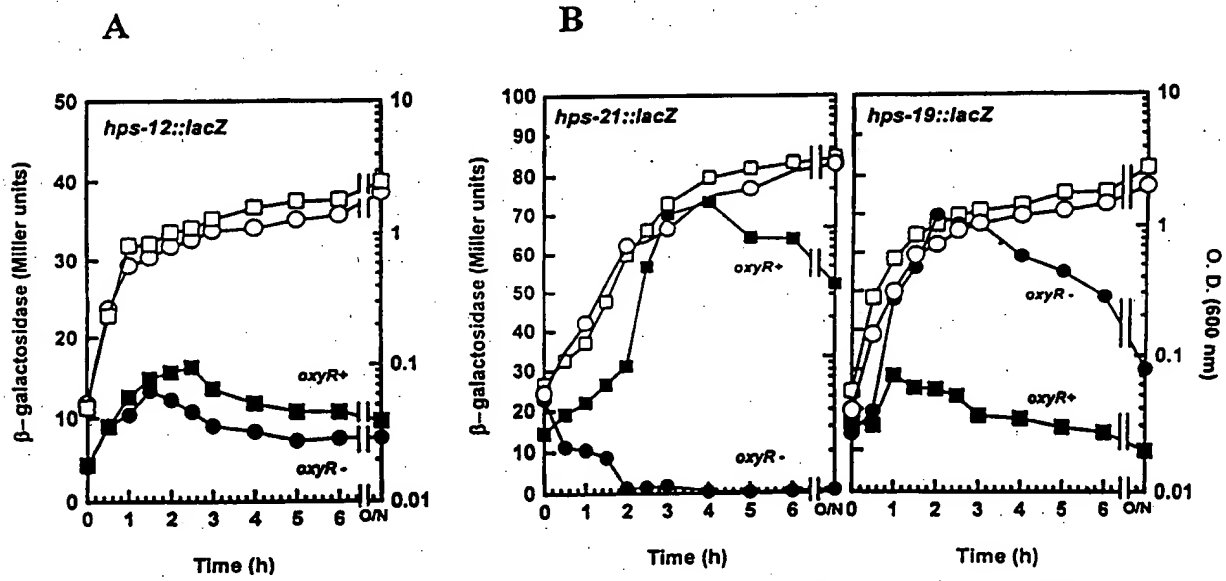

FIG. 2. Expression of *hps::lacZ* fusions in wild-type and Δ*oxyR* backgrounds. (A) Representative class I fusion mutant showing an OxyR-independent expression pattern during growth in LB medium in wild-type and Δ*oxyR* backgrounds. (B) OxyR-dependent expression. The expression of the *hps-21::lacZ* fusion was very low in the absence of OxyR; hence, this fusion is a class II (OxyR-activated) fusion mutant. *hps-19::lacZ* expression was repressed in the presence of OxyR; thus, this fusion is a class III fusion mutant. Symbols: ■ and ●, expression of the *lacZ* fusion in wild-type and Δ*oxyR* backgrounds, respectively; □ and ○, growth (expressed in OD_{600}) for wild-type and Δ*oxyR* strains, respectively. O/N, overnight.

TABLE 5. Complementation of *hps* mutants by a plasmid-borne *oxyR* gene under an IPTG-inducible *tac* promoter[a]

Allele	Class	β-Galactosidase activity (Miller units)				
		Wild type	ΔoxyR			
			Without vector	Plasmids only	pAQ25 (*oxyR*)	
					− IPTG	+ IPTG[b]
OxyR independent, *hps-11*	I	124.0	138.0	139.0	130.0	136.0
OxyR activated						
hps-2	II	109.0	36.6	34.0	64.8	131.5 (3.6)[c]
hps-4	II	60.5	34.4	38.8	63.4	72.8 (2.1)
hps-5	II	40.9	27.6	28.8	21.1	47.4 (1.7)
hps-10	II	103.6	40.5	31.9	84.2	134.1 (3.3)
hps-14	II	78.7	45.9	50.5	87.9	165.8 (3.6)
hps-16	II	91.6	49.3	50.5	77.4	147.5 (3.0)
hps-20	II	65.0	27.2	34.6	96.0	146.4 (5.4)
hps-21	II	59.0	6.0	5.8	20.3	84.7 (14.1)
hps-24	II	35.4	29.1	28.4	62.7	92.0 (3.2)
hps-27	II	105.0	35.1	31.9	84.3	219.0 (6.2)

[a] All cultures were serially subcultured twice to an OD_{600} of 0.2. Cultures containing pAQ25 transformants were divided into two equal portions, and to one portion IPTG was added to a final concentration of 1 mM. Cultures were incubated at 37°C at 200 rpm. Aliquots of cultures were removed periodically to assay β-galactosidase activities. Data are for aliquots taken 90 min after addition of IPTG. Addition of 1 mM IPTG to cultures of wild-type, Δ*oxyR*, and Δ*oxyR*/pKK177-3 (vector only) strains did not alter the basal level of β-galactosidase activity relative to that of an untreated culture. Only one member of the OxyR-independent group has been included here. All other members of this group exhibited similar levels of expression in both *oxyR*$^+$ and Δ*oxyR* backgrounds.

[b] 1 mM (final concentration).

[c] Fold induction relative to that of an Δ*oxyR* strain under similar conditions.

from the translational start site, disrupting the codon for Val 166 (50). Similarly, in the *hps-5*::*lacZ* mutant, the insertion is in the noncoding strand of the *thiG* gene, disrupting the codon for Ala 146 (47), and in *hps10*::*lacZ*, the insertion is in the noncoding strand, disrupting the codon for Arg 90 (5).

The fusion in *hps-21* is in an open reading frame (ORF), *f497* (8, 38), whose probable translation product is similar to arylsulfatase enzymes and is close to the *oriC* region of the chromosome that initiates replication. Although different promoter elements in this region have been mapped (20), we do not know the ones that are driving the expression of the fusion. The fusion in the *hps-4*::*lacZ* mutant is in the *hemF* gene, which encodes the aerobically produced coproporphyrinogen III oxidase involved in heme biosynthesis (45). The *hps-2* mutation maps to the noncoding strand of cell division gene *ftsJ* (1). However, the bases immediately following the fusion junctions in these mutants do not correspond to the published sequence information. The discrepancy in sequence information can be resolved by cloning the fusion junctions by the mini-Mu method (27) and comparing them with the sequences obtained by the UV method (33).

Sensitivity of *hps-24* to mitomycin. Strains with mutations in the *uvrD* gene, whose product is a DNA helicase II involved in nucleotide excision repair and mismatch repair of DNA, are sensitive to mitomycin (an interstrand linking agent causing replication blocks). We compared the sensitivity of strain RK724, containing *hps-24* (*lacZ* inserted in the *uvrD* gene), to

TABLE 6. Effects of hydrogen peroxide on the expression of OxyR-activated and hydrogen peroxide-inducible *hps*::*lacZ* fusions during exponential phase[a]

Strain	*lacZ* fusion	β-Galactosidase activity (Miller units)		Fold induction
		Without H_2O_2	With H_2O_2	
RK4936/pAQ24	*katG*::*lacZ*	1,800	3,300	1.8
RK702	*hps-2*::*lacZ*	100	161	1.6
RK704	*hps-4*::*lacZ*	6.5	11.9	1.8
RK710	*hps-10*::*lacZ*	52.2	135.3	2.5
RK716	*hps-16*::*lacZ*	8.8	43.2	4.9
RK720	*hps-20*::*lacZ*	45.7	68.4	1.5
RK721	*hps-21*::*lacZ*	13.5	23.3	1.8
RK727	*hps-27*::*lacZ*	31.7	88.1	2.8

[a] Overnight cultures of strains were subcultured twice to an OD_{600} of 0.2, divided into two portions, and inoculated into flasks containing prewarmed LB to an initial OD_{600} of 0.1. To one flask, hydrogen peroxide was added to a final concentration of 60 μM; both flasks were incubated for 60 min at 200 rpm and 37°C. After 60 min, cultures were placed on ice, and chloramphenicol (150 μg/ml [final concentration]) was added to stop further protein synthesis. Subsequently, cultures were assayed for β-galactosidase activity.

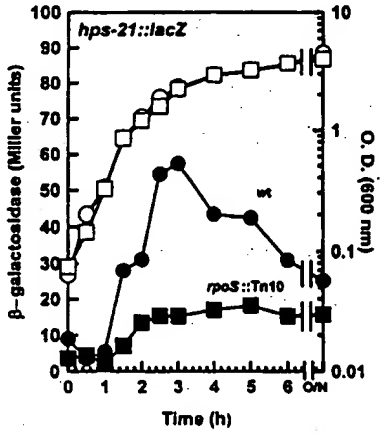

FIG. 3. RpoS-dependent expression of *hps-21*::*lacZ* during growth. The β-galactosidase activities of the *hps-21*::*lacZ* fusion in isogenic wild-type (*wt*) and *rpoS*::Tn*10* backgrounds were determined in LB medium during growth, as described in Materials and Methods. Symbols: ■ and ●, β-galactosidase activities in *rpoS* and wild-type backgrounds, respectively; □ and ○, growth (expressed in OD_{600}) for wild-type and *rpoS* strains, respectively. O/N, overnight.

TABLE 7. Regulation of OxyR-activated genes by RpoS[a]

Allele	Growth phase[b]	β-Galactosidase activity (Miller units)		Ratio
		rpoS⁺	*rpoS*	
hps-5	Exponential	12.8	16.0	
	Early stationary	50.3	26.1	2
hps-19	Exponential	10.8	7.6	
	Early stationary	37.3	3.8	10
hps-21	Exponential	3.6	4.5	
	Early stationary	54.4	15.4	3.5

[a] Overnight cultures were serially subcultured twice to an OD_{600} of 0.2 and inoculated into LB media to an initial OD_{600} of 0.05. Aliquots of cultures were removed every 30 min and assayed for β-galactosidase activity.

[b] Exponential phase refers to cultures at an OD_{600} of 0.2, growing for 30 min after subculture; early stationary phase refers to cultures at an OD_{600} of 2.0, growing for 2.5 h after subculture.

mitomycin relative to that of another nonisogenic *uvrD* mutant, HS6882 (Table 9). The *hps-24* mutant was sensitive to mitomycin compared to the parent strains, and the degree of sensitivity was similar to those of strains HS008 (wild type) and HS6882 (*uvrD*::Tn*5*) (both sets exhibited a 3-mm increase in the radius of inhibition of the mutant strain).

DISCUSSION

It is known that oxidative stress (hydrogen peroxide treatment) induces the synthesis of many protein in *S. typhimurium* (10, 28). However, only a few of the genes expressing the above-mentioned proteins have been identified. From a random chromosomal fusion library, 26 mutants that exhibited a hydrogen peroxide-sensitive phenotype reproducibly in three different genetic backgrounds (MC4100, GC4468, and RK4936) were isolated. Since transposon insertions were used to generate these fusions, our screen may have missed the identification of genes essential for survival during hydrogen peroxide-stressed conditions (as mutations in these genes will not be viable) or during growth. However, we have identified a set of nonessential genes that play a significant role in cell survival against oxidative damage.

These genes were grouped into three major classes based on expression pattern of the *hps*::*lacZ* fusions during growth, hydrogen peroxide inducibility, and complementation by a plasmid-borne copy of the *oxyR* gene (Table 4). The expression of the *lacZ* fusion of class I mutants was independent of OxyR regulation. A mutation in the *xthA* gene, encoding exonuclease III, which is involved in the repair of damaged DNA, renders the cell sensitive to hydrogen peroxide (15) and is similar to our class I mutants. The hydrogen peroxide- and heat shock-inducible *dnaK* gene product, DnaK, which is involved in the initiation of DNA replication, may play a role in the macromolecular assemblies of proteins under oxidative stress as is evident from its similarity to the *groE* gene product, which is involved in encoding molecular chaperones like GroEL and GroES proteins. Mutants deficient in *dnaK*, like class I *hps* mutants, are hydrogen peroxide sensitive (9).

Class II *hps* genes require functional OxyR protein for maximum expression. Some class II mutants, which are activated by OxyR, showed increased levels of expression at the onset of stationary phase (Fig. 3). It has been recently shown not only that *katG* (23, 29), *dps* (3), and *gorA* (4) are members of the OxyR regulon but that their growth phase expression is regulated by RpoS, the second principal sigma factor responsible for starvation stress-induced gene expression (24, 26, 41). The expression of *katG* (encoding HPI hydroperoxidase) increases to a maximum as the cultures enter into stationary phase and is regulated at the level of transcription by RpoS; however, the hydrogen peroxide inducibility is regulated in exponential-phase cultures at the level of transcription by OxyR (10, 28, 29). We have identified two genes among class II (OxyR-activated) mutants (*hps-5* and *hps-21*) and one among class III mutants (*hps-19*) that show dual regulation.

One of the identified fusions of the OxyR-activated class mapped to *rcsC*, a probable membrane-bound signal transducer of a two-component regulatory network that controls capsular polysaccharide biosynthesis (40). Like mutations affecting other sensor transducers, such as *ntrB* (30), mutations in *rcsC* do not appear to confer a distinguishing phenotype (40). However, it is possible that a cell that is defective in capsule synthesis is vulnerable to some exogenous membrane-damaging agents. This may explain why an *rcsC* mutant, defective in expressing the *cps* capsular polysaccharide biosynthesis genes, is hydrogen peroxide sensitive. The lack of an observable phenotype for *rcsC* mutants may be partially due to an overlap in genetic regulation. For example, cross talk between distinct systems, such as those that regulate nitrogen assimilation and chemotaxis (30), may suppress the effect of a

TABLE 8. Locations of the *lacZ* fusion junctions in OxyR-activated *hps* mutants that map to known *E. coli* genes

Fusion	Chromosomal location (min)	Gene[a]	Function (reference[s])
hps-2	67.4	*ftsJ*[b]	Cell division gene (1)
hps-4	50	*hemF*[c]	Aerobically produced coproporphyrinogen III oxidase; required for synthesis of protoheme IX (45)
hps-5	89	*thiG*[d]	Required for synthesis of thiazole in thiamine synthesis (5, 47)
hps-10	89	*gldA*[e]	Glycerol dehydrogenase (5, 46)
hps-21	81.5	*f497*	Similar to arylsulfatase; unknown function (8, 38)
hps-24	86	*uvrD*[f]	DNA-dependent ATPase and DNA helicase II (13, 50)
hps-27	48	*rcsC*	Sensor-regulator protein of capsular polysaccharide-synthesizing genes (50)

[a] Locations of *lacZ* fusion junctions within known sequences were identified from Blast searches (2).

[b] The insertion is in opposite orientation relative to transcription of the *ftsJ* gene. We are presently unable to identify the potential promoter element driving the expression of the integrated *lacZ* gene from information obtained from GenBank entry ECOUW67.

[c] *E. coli* gene with maximum homology to our sequence data.

[d] The insertion is in opposite orientation from transcription of the *thiG* gene. The potential promoter element driving the transcription of the integrated *lacZ* gene may be that of the *htrC* gene from information obtained from GenBank entry ECOUW89 (5).

[e] The insertion is in opposite orientation from transcription of the *gldA* gene. We are presently unable to identify potential promoter elements driving the expression of the integrated *lacZ* gene from information obtained from GenBank entry ECOUW89 (5).

[f] The insertion is in opposite orientation from transcription of the *uvrD* gene. The potential promoter element driving the transcription of the integrated *lacZ* gene may be that of either ORF *f125* or ORF *f161* from information obtained from GenBank entry X00738 (13).

TABLE 9. Sensitivity of HS724 (*uvrD::lacZ*) to mitomycin

Strain	Relevant genotype	Radius of inhibition (mm)[a]
RK4936	Wild type	5.5
HS008	Wild type	7.5
TA4112	Δ*oxyR*	5.5
HS6882	*uvrD*::Tn*5*	10.5
RK724	*uvrD::lacZ*	8.2
TA724	Δ*oxyR uvrD::lacZ*	8.8

[a] A 0.2-ml aliquot of a culture grown for 6 h was plated on an LB plate with 3 ml of soft agar. Filter discs containing mitomycin (10 μl from a 2-mg/ml stock) were placed on the middle of each overlaid plate and incubated for 12 h at 37°C. Each value is the average of triplicate assays. The standard error for each set of experiments was less than 0.2 mm.

mutation that affects a given two-component regulator. Determinations of levels of catalase expression, particularly that of HPI hydroperoxidase, in other *rcsC* mutants may help resolve this issue.

The *hps-21::lacZ* fusion mutant exhibited dual regulation by RpoS and OxyR. The fusion was mapped to an ORF, *f497* (8, 38), whose product is similar to arylsulfatase enzymes (Table 8). Human intestinal micro flora uses arylsulfatase enzymes to break down steroid sulfates in the bile acid and subsequently uses the sulfur for dissimilation (48), while the steroid backbone is reabsorbed by the intestine (18). Often intestinal microflora uses chondroitin sulfate, a mucopolysaccharide of the intestinal tissue, as the sole carbohydrate source (34). The breakdown of chondroitin sulfate to unsulfated di- and monosaccharides is catalyzed by bacterial sulfatases (34). Since the functions of these sulfatases are required under conditions of carbon starvation, it is not surprising that their expression is σ^S dependent (Fig. 3), the sigma factor that directs RNA polymerase to promoters of genes required for survival during starvation. The genes near the bacterial origin of replication are highly conserved, and their functions may play an important role during cell growth (31); however, the independent regulation of a putative metabolic gene by OxyR is unclear at this time, and further studies are required.

One of the *hps* genes showing twofold hydrogen peroxide inducibility and OxyR dependence (complementation with plasmid-borne *oxyR*), *hps-4* (Table 8), was identified as the *hemF* gene, encoding an aerobically induced enzyme, coproporphyrinogen III oxidase, in protoheme IX biosynthesis (45). Protoheme IX is required for activity of both HPI(12) and HPII—the latter as heme d, a *cis*-hydroxylated protoheme IX derivative (25). When cultures are exposed to hydrogen peroxide in exponential phase, there is a rapid increase in *katG* (structural gene for HPI hydroperoxidase) transcription (28, 39). However, to form a functional HPI hydroperoxidase to deal with the challenge, the cell needs to synthesize an adequate amount of protoheme IX. A deficiency is expected to render the cell vulnerable to hydrogen peroxide challenge, as observed for the *hps-4* mutant (Fig. 1). Early-stationary-phase cultures of the *hps-4::lacZ* mutant strain had significantly lower levels of both HPI and HPII catalase expression compared to those of an isogenic wild-type strain, as determined by catalase zymograms (35a). The residual catalase activity in this mutant strain is probably due to the synthesis of protoheme IX via the alternate biosynthetic pathway. Since the *hemF* gene product is required for functional HPI catalase synthesis, it is not surprising that *hemF* and *katG* are regulated by OxyR. Thus, *hemF* is also a member of the OxyR regulon.

We presume that the *lacZ* transcription in fusions that are opposite in orientation to the known pr moters are probably driven by promoterlike elements in the 3′ regions of the known genes. Although the ORF causing the Hps phenotype in *hps-5* (*thiG*) is yet to be determined (5), it is possible that this fusion is driven by the promoter of *htrC*, a heat shock-inducible gene (32). Our speculation is based on the fact that five proteins, which are members of the hydrogen peroxide regulon, show elevated levels of expression under conditions of heat shock (28). Of these five overexpressing proteins (F52a, E89, D64a, C69, and E79), three are regulated by OxyR (F52a, E89, and D64a) and the identities of only two members are known (F52a, a component of alkyl hydroperoxidase reductase; and C69, DnaK protein). Alkyl hydroperoxidase breaks down hydrogen peroxide, while DnaK may be involved in the repair of peroxidatively damaged DNA. It may also be speculated that DnaK protein, in its capacity as a molecular chaperone, is involved in proper protein folding under oxidizing conditi ns within the cell.

It is not clear which promoter is involved in the expression of the *lacZ* fusion in *hps-24::lacZ* (noncoding strand of *uvrD*); the probable promoter(s) driving the expression may be that f either ORF *f125* or *f161* (13). It is also possible that the insertions in the noncoding strands of known genes cause a polar effect, thereby preventing the expression of essential genes located on the 3′ end and resulting in the hydrogen peroxide-sensitive phenotype.

Our screen failed to identify any previously known OxyR-regulated genes. Since we screened for *lac*$^+$ fusion mutants that were hydrogen peroxide sensitive, our screen would n t detect any insertion opposite in orientation to an OxyR-regulated promoter. Thus, a hydrogen peroxide-sensitive phenotype caused by an insertional mutation in opposite orientation to any known OxyR-regulated genes would not be detected. In the largely uncharacterized OxyR-independent fusion mutants (class I), some of the insertions may have taken place in known OxyR-regulated genes but in opposite orientation to the OxyR-regulated promoter, resulting in a hydrogen peroxide phenotype where the expression of the *lacZ* gene is directed by an unrelated OxyR-independent promoter element. These questions can be addressed with further characterization f class I fusion mutants.

Class III fusion mutants showed increased *lacZ* expression in an Δ*oxyR* background. The expression pattern of the *hps-19::lacZ* mutant suggests that the promoter element(s) is recognized by both RpoS and OxyR and that OxyR acts as a repressor, as is evident from its own expression (10, 44). The class III fusions indicate that OxyR acts as a repressor and represses a group of genes in *E. coli*.

We have attempted to identify the members of the regul n that are induced when *E. coli* is subjected to oxidative stress generated by exposure to hydrogen peroxide and that have protective functions within the cell. Our approach has identified not only additional members of the OxyR-activated regulon but also members of an as-yet-unknown OxyR-repressed regulon.

ACKNOWLEDGMENTS

We thank G. Storz for *oxyR* plasmid constructs and bacterial strains and for bearing with our repeated requests. We also thank D. Walker and L. Wei for screening the fusion bank for hydrogen peroxide-sensitive mutants, R. N. Roy for assistance in DNA sequencing, and C. Palmer and D. Serafini for critically reviewing the manuscript.

This work was supported by an operating grant to H.E.S. from the National Sciences and Engineering Research Council (NSERC) of Canada. S.M. was supported, in part, by a Canadian International Development Agency scholarship.

REFERENCES

1. Aldea, M., C. Hernandez-Chico, A. G. de la Campa, S. K. Kushner, and M. Vincent. 1988. Identification, cloning, and expression of *bolA*, an *ftsZ*-dependent morphogenes of *Escherichia coli*. J. Bacteriol. 170:5169–5170.
2. Altschul, S. F., W. Gish, W. Miller, E. W. Myer, and D. J. Lipman. 1990. Basic logic alignment search tool. J. Mol. Biol. 215:403–410.
3. Altuvia, S., M. Almiron, G. Huisman, R. Kolter, and G. Storz. 1994. The *dps* promoter is activated by OxyR during growth and by IHF and σ^S in stationary phase. Mol. Microbiol. 13:265–272.
4. Becker-Hapak, M., and A. Eisenstark. 1995. Role of *rpoS* in the regulation of glutathione oxidoreductase (*gor*) in *Escherichia coli*. FEMS Microbiol. Lett. 134:39–44.
5. Blattner, F. R., V. Burland, G. Plunkett III, H. J. Sofia, and D. L. Daniels. 1993. Analysis of the *Escherichia coli* genome. IV. DNA sequence of the region from 89.2 to 92.8 minutes. Nucleic Acids Res. 21:5408–5417.
6. Bölker, M., and R. Khamann. 1989. The *Escherichia coli* regulatory protein OxyR discriminates between methylated and unmethylated states of the phage Mu *mom* promoter. EMBO J. 8:2403–2410.
7. Bremer, E., T. J. Silhavy, and G. M. Weinstock. 1985. Transposable λ*plac*Mu bacteriophages for creating *lacZ* operon fusions and kanamycin resistance insertions in *Escherichia coli*. J. Bacteriol. 162:1092–1099.
8. Burland, V., G. Plunkett III, D. L. Daniels, and F. R. Blattner. 1993. DNA sequence and analysis of 136 kilobases of *Escherichia coli* genome: organizational symmetry around the origin of replication. Genomics 16:551–561.
9. Caron, E., M. Cellier, J. P. Liautar, and S. Kohler. 1994. Complementation of a DnaK-deficient *Escherichia coli* strain with *dnaKJdnaJ* operon of *Brucella ovis* reduces the rate of initial intracellular killing within monocytic cell line U937. FEMS Microbiol. Lett. 120:335–340.
10. Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell 41:753–762.
11. Christman, M. F., G. Storz, and B. N. Ames. 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA 86:3484–3488.
12. Claiborne, A., and I. Fridovich. 1979. Purification of the o-dianisidine peroxidase from *Escherichia coli* B. J. Biol. Chem. 254:4245–4252.
13. Daniels, D. L., G. Plunkett III, V. Burland, and F. R. Blattner. 1992. Analysis of the *Escherichia coli* genome: DNA sequences of the region from 84.5 to 86.5 minutes. Science 257:771–777.
14. Demple, B., and J. Halbrook. 1983. Inducible repair of oxidative damage in *Escherichia coli*. Nature 304:466–468.
15. Demple, B., J. Halbrook, and S. Linn. 1983. *Escherichia coli xth* mutants are hypersensitive to hydrogen peroxide. J. Bacteriol. 153:1079–1082.
16. Demple, B., and L. Harrison. 1994. Repair of oxidative damage to DNA: enzymology and biology. Annu. Rev. Biochem. 63:915–948.
17. Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and G. G. Guiney. 1992. The alternative σ factor KatF (RpoS) regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. USA 89:11978–11982.
18. Huijghebaert, S., G. Parmentier, and H. Eyssen. 1984. Specificity of bile salt sulfatase in man, mouse and rat intestine microflora. J. Steroid Biochem. 20:907–912.
19. Imlay, J. A., and S. Linn. 1986. Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. J. Bacteriol. 166:519–527.
20. Kölling, R., A. Gielow, W. Seufert, C. Kücherer, and W. Messer. 1988. AsnC, a multifunctional regulator of genes located around the replication origin of *Escherichia coli*, *oriC*. Mol. Gen. Genet. 212:99–104.
21. Kullik, I., M. B. Toledano, L. A. Tartaglia, and G. Storz. 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. J. Bacteriol. 177:1275–1284.
22. Kullik, I., J. Stevens, M. B. Toledano, and G. Storz. 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. J. Bacteriol. 177:1285–1291.
23. Ivanova, A., C. Miller, G. Glinsky, and A. Eisenstark. 1994. Role of *rpoS* (*katF*) in *oxyR*-independent regulation of hydroperoxidase I in *Escherichia coli*. Mol. Microbiol. 12:571–587.
24. Lange, R., and R. Hengge-Aronis. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. 5:49–59.
25. Loewen, P. C., J. Switala, I. von Ossowski, A. Hiller, A. Christie, B. Tattrie, and P. Nicholls. 1993. Catalase HPII of *Escherichia coli* catalyses the conversion of protoheme to *cis*-heme d. Biochemistry 32:10159–10164.
26. McCann, M. P., J. P. Kidwell, and A. Matin. 1991. The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. J. Bacteriol. 173:4188–4194.
27. Miller, J. H. 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. N. Ames. 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. USA 83:8059–8063.
29. Mukhopadhyay, S., and H. E. Schellhorn. 1994. Induction of *Escherichia coli* hydroperoxidase I by acetate and other weak acids. J. Bacteriol. 176:2300–2307.
30. Ninfa, A. J., E. G. Ninfa, A. N. Lupas, A. Stock, B. Magasanik, and J. Stock. 1988. Crosstalk between bacterial chemotaxis signal transduction proteins and regulators of transcription of Ntr regulon: evidence that nitogen assimilation and chemotaxis are controlled by a common phosphotransfer mechanism. Proc. Natl. Acad. Sci. USA 85:5492–5496.
31. Ogasawara, N., and H. Yoshikawa. 1992. Genes and their organization in the replication origin region of bacterial chromosome. Mol. Microbiol. 6:629–634.
32. Raina, S., and C. Georgopoulos. 1990. A new *Escherichia coli* heat shock gene, *hsrC*, whose product is essential for viability only at high temperature. J. Bacteriol. 172:3417–3426.
33. Roy, R. N., S. Mukhopadhyay, L. L-C. Wei, and H. E. Schellhorn. 1995. Isolation and sequencing of gene fusions carried by λ*plac*Mu specialized transducing phage. Nucleic Acids Res. 23:3076–3078.
34. Salyers, A. A., and M. O'Brien. 1980. Cellular location of enzymes involved in chondroitin sulfate breakdown by *Bacteroides thetaiotaomicron*. J. Bacteriol. 143:772–780.
35. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

35a. Schellhorn, H. E. Unpublished data.

36. Schellhorn, H. E., and V. L. Stones. 1992. Regulation of *katF* and *katE* in *Escherichia coli* K-12 by weak acids. J. Bacteriol. 174:4769–4776.
37. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. Sofia, J. H., V. Burland, D. L. Daniels, G. Plunkett III, and F. R. Blattner. 1994. Analysis of the *Escherichia coli* genome. V. DNA sequence of the region from 76.0 to 81.5 minutes. Nucleic Acids Res. 22:2576–2586.
39. Storz, G., L. A. Tartaglia, and B. N. Ames. 1990. Transcription regulator of oxidative stress-inducible genes: direct activation by oxidation. Science 248:189–194.
40. Stout, V., and S. Gottesman. 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. 172:659–669.
41. Tanaka, K., Y. Takayanagi, N. Fujita, A. Ishihama, and H. Takahashi. 1993. Heterogeneity of principal σ factor of *Escherichia coli*: the *rpoS* gene product, σ^{38}, is the second principal σ factor of RNA polymerase in stationary-phase *Escherichia coli*. Proc. Natl. Acad. Sci. USA 90:3511–3515.
42. Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa. 1991. Purification and characterization of the *Escherichia coli* OxyR protein, a positive regulator for a hydrogen peroxide-inducible regulon. J. Biochem. 109:262–266.
43. Tartaglia, L. A., G. Storz, and B. N. Ames. 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for bacterial adaptation to oxidative stress. J. Mol. Biol. 210:709–719.
44. Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz. 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA binding site: a mechanism for differential promoter selection. Cell 78:897–909.
45. Troup, B., M. Jahn, C. Hungerer, and D. Jahn. 1994. Isolation of the *hemF* operon containing the gene for the *Escherichia coli* aerobic coproporphyrinogen III oxidase by in vivo complementation of a yeast *HEM13* mutant. J. Bacteriol. 176:673–680.
46. Truniger, V., and W. Boos. 1994. Mapping and cloning of *gldA*, the structural gene of the *Escherichia coli* glycerol dehydrogenase. J. Bacteriol. 176:1796–1800.
47. Van der Horn, P. B., A. D. Backstrom, V. Stewart, and T. P. Begley. 1993. Structural genes for thiamine biosynthetic enzymes *thiCEFGH* in *Escherichia coli* K-12. J. Bacteriol. 175:982–992.
48. Van Eldere, J., G. Parmentier, S. Asselberghs, and H. Eyssen. 1991. Partial characterization of steroidsulfatases in *Peptococcus niger* H4. Appl. Environ. Microbiol. 57:69–71.
49. Winquist, L., U. Rannug, A. Rannug, and C. Ramel. 1984. Protection from toxic and mutagenic effects of hydrogen peroxide by catalase induction in *Salmonella typhimurium*. Mutat. Res. 141:145–147.
50. Yamamoto, Y., T. Ogawa, H. Shinagawa, T. Nakayama, H. Matsuo, and H. Ogawa. 1986. Determination of the initiation sites of transcription and translation of the *uvrD* gene of *Escherichia coli*. J. Biochem. 99:1579–1590.

JOURNAL OF BACTERIOLOGY, Aug. 1999, p. 4639–4643
0021-9193/99/$04.00+0

Vol. 181, No. 15

OxyR and SoxRS Regulation of *fur*

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Received 9 April 1999/Accepted 21 May 1999

The cytotoxic effects of reactive oxygen species are largely mediated by iron. Hydrogen peroxide reacts with iron to form the extremely reactive and damaging hydroxyl radical via the Fenton reaction. Superoxide anion accelerates this reaction because the dismutation of superoxide leads to increased levels of hydrogen peroxide and because superoxide elevates the intracellular concentration of iron by attacking iron-sulfur proteins. We found that regulators of the *Escherichia coli* responses to oxidative stress, OxyR and SoxRS, activate the expression of Fur, the global repressor of ferric ion uptake. A transcript encoding Fur was induced by hydrogen peroxide in a wild-type strain but not in a Δ*oxyR* strain, and DNase I footprinting assays showed that OxyR binds to the *fur* promoter. In cells treated with the superoxide-generating compound paraquat, we observed the induction of a longer transcript encompassing both *fur* and its immediate upstream gene *fldA*, which encodes a flavodoxin. This polycistronic mRNA is induced by paraquat in a wild-type strain but not in a Δ*soxRS* strain, and SoxS was shown to bind to the *fldA* promoter. These results demonstrate that iron metabolism is coordinately regulated with the oxidative stress defenses.

Reactive oxygen species can damage DNA, lipid membranes, and proteins and have been implicated in numerous diseases. As a defense, both prokaryotic and eukaryotic cells have inducible responses that protect against oxidative damage. These antioxidant defense systems have been best characterized in *Escherichia coli* (reviewed in reference 29). Hydrogen peroxide activates the transcription factor OxyR through the oxidation of two cysteines and formation of an intramolecular disulfide bond (34). Activated OxyR then induces transcription of a set of antioxidant genes, including *katG* (hydroperoxidase I), *ahpCF* (alkylhydroperoxidase), *dps* (a nonspecific DNA binding protein), *gorA* (glutathione reductase), *grxA* (glutaredoxin I), and *oxyS* (a regulatory RNA). Superoxide-generating compounds, such as paraquat, activate the transcription factor SoxR by oxidizing the 2Fe-2S cluster in the protein through an unknown mechanism (11, 16). Oxidized SoxR then induces the expression of the second transcription factor SoxS, which directly activates the transcription of *sodA* (manganese superoxide dismutase), *fpr* (ferredoxin/flavodoxin-$NADP^+$ reductase), *zwf* (glucose 6-phosphate dehydrogenase), *fumC* (fumarase C), *nfo* (endonuclease IV), *acnA* (aconitase A), and *micF* (a regulatory RNA).

There is an intimate relationship between iron metabolism and oxidative stress. Iron is an indispensable element for living cells, since many metabolic enzymes have iron as a cofactor in their active sites. On the other hand, through the Fenton reaction, iron also promotes the formation of hydroxyl radicals, which indiscriminately damage all cellular components. Thus, cells have evolved regulatory systems to ensure the sufficient uptake of iron to meet their physiological requirements yet at the same time minimize iron toxicity. The regulation of iron homeostasis in both eukaryotic and prokaryotic cells is the subject of intense study, and much is known. In prokaryotic cells, a transcription factor denoted Fur (ferric uptake regulation) negatively regulates many genes involved in ferric iron uptake from the environment (reviewed in references 6 and 7). Most Fur-regulated genes are derepressed in growth at low iron and are repressed under conditions of high iron, and in vitro DNA binding assays suggest that high levels of iron favor Fur association with DNA (2, 3, 9, 15). Thus, Fur is considered to be an iron-dependent repressor. The findings that Δ*fur* mutants are sensitive to hydrogen peroxide and show increased oxidative DNA damage and mutations under aerobic conditions have implicated Fur in the defenses against oxidative stress (32). However, Fur expression in response to oxidative stress has not been examined.

As part of our continuous effort to better define the physiological role of OxyR, we initiated a computational approach to identify additional OxyR-regulated genes. We used an algorithm based on information theory (26) that uses previously identified OxyR binding sites as a model to search through the entire *E. coli* genome for new OxyR binding sites. This approach predicted an OxyR binding site in the promoter region of the *fur* gene. Subsequent experiments confirmed OxyR binding to this region and showed that *fur* expression is induced by OxyR after hydrogen peroxide treatment. We also found that SoxRS regulates *fur* by activating the expression of a transcript encoding both flavodoxin and Fur. These results show that the control of iron metabolism in *E. coli* is an integral part f the antioxidant defense response and that the regulation of Fur by OxyR and SoxRS directly reflects the chemistry between iron and reactive oxygen species.

MATERIALS AND METHODS

Computer search program. The seven OxyR target sequences analyzed previously (25) and two new sites (*grxA* at position 207 in GenBank entry M13449 and a second Mu phage *mom* site at position 59 in GenBank entry V01463) were used to generate an individual information weight matrix (26). The matrix was scanned across the entire *E. coli* genomic sequence (5). The sites identified were sorted by information content so that the strongest sites could be investigated further. Local regions of the genome surrounding the strongest sites were displayed using the Lister program (version 9.02) (Fig. 1) to show coding regions along with sequence walkers representing potential binding sites (27). Detailed Lister maps of the *fldA-fur* region including ribosome binding sites and cyclic AMP receptor protein sites are available online (28).

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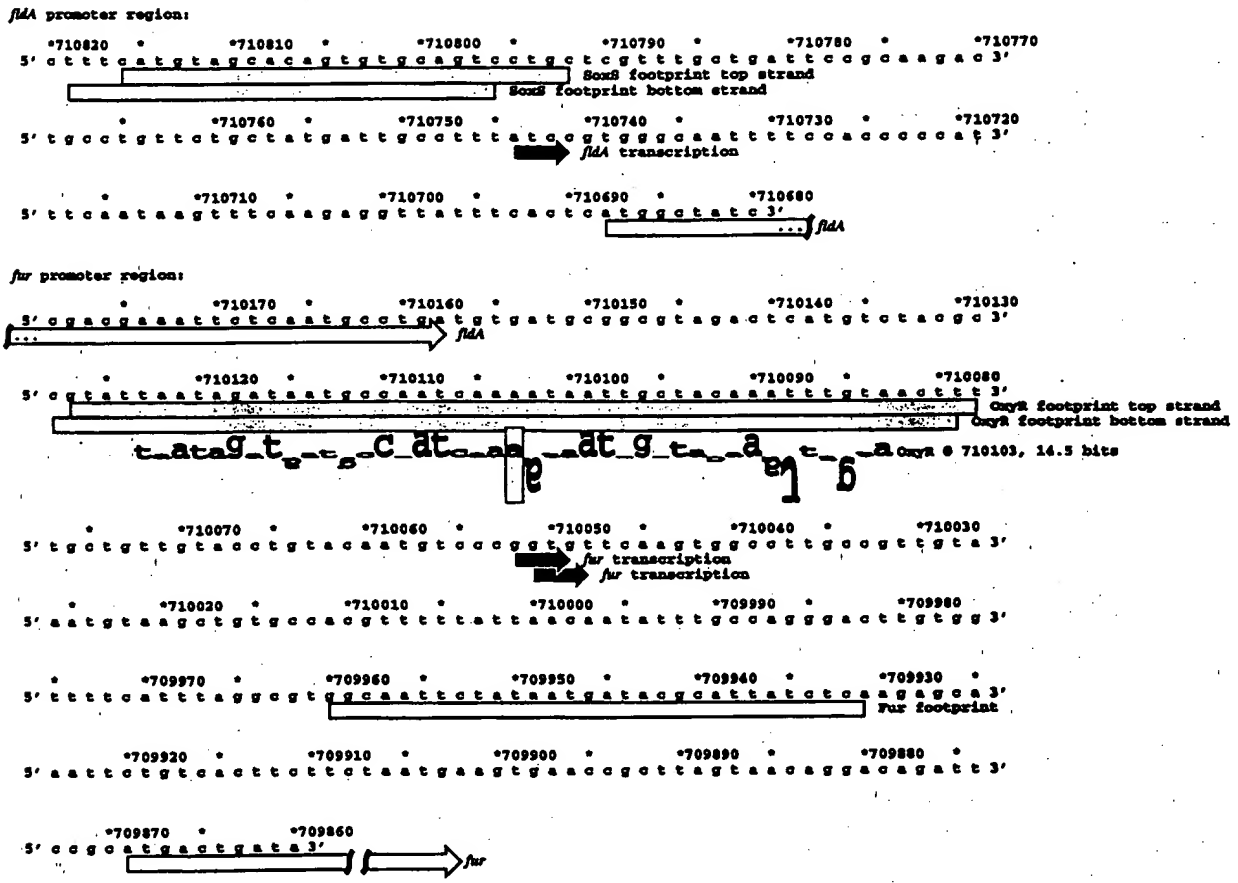

FIG. 1. Sequences of *fur* and *fldA* promoter regions. The DNA sequences and coordinates are for *E. coli* from GenBank accession no. U00096 (5). The transcriptional starts are marked by black arrows, the *fldA* and *fur* open reading frames are indicated by white arrows, and the DNase I footprints are denoted by gray boxes. The location of the predicted OxyR site is shown by a sequence walker (27), in which the rectangle surrounding the adenine at position 710103 indicates the center (zero base) of the binding site. A sequence walker consists of a string of letters in which the height of each letter shows the contribution that the corresponding base would make to the average sequence conservation shown by the sequence logo of all binding sites (25). The sequence walker is given on a scale of bits of information. The rectangle surrounding the adenine gives the scale, from −3 bits up to the maximum conservation at +2 bits. Positively contributing bases are above the zero line, while negatively contributing ones are below the line. By using bits, the heights of all letters can be added together to obtain the total sequence conservation of a site. The *fur* site is 14.5 bits, and the average OxyR site is 16.7 ± 1.9 bits. The rectangle surrounding the inverted thymine at position 710088 indicates that no T residues were found at this position in the OxyR sites used to construct the model.

Strains and plasmids. The plasmids used in this study were constructed as follows. An 840-bp fragment carrying the *fldA* gene was amplified by PCR from genomic DNA (using the primers 5′-GCC ACT TGA ATT CCG GGA CAT TG and 5′-GAA CGG ATC CAA GAG ATG TTA ATG C [underlined sequences are restriction sites]) and cloned into the *Eco*RI and *Bam*HI sites of pUC18 to generate pGSO96. A 250-bp fragment containing the *fur* promoter region was amplified by PCR from genomic DNA (using primers 5′-AAT GAA TTC CAC AAG TCC CTG GC and 5′-CCG CGG ATC CTG TAG AAA AAT GGG) and cloned into the *Eco*RI and *Bam*HI sites of pUC18 to generate pGSO97. A 200-bp fragment containing the *fldA* promoter was PCR amplified from genomic DNA (using primers 5′-GCC GTA GCG AAC GGA TCC AAG AG and 5′-GCC AGT GAA AGC TTT GAG TG) and subcloned into the *Hin*dIII and *Bam*HI sites of pUC18 to generate pGSO98. The integrity of all clones was verified by DNA sequencing. Standard cloning techniques were used. The Δ*oxyR*::*kan* (GSO9 [32]), Δ*soxRS-zjc2205 zjc2204*::Tn*10kan* (DJ901 [14]), and Δ*fur*::*kan* (QC1732 [32]) mutant alleles were moved into MC4100 by P1 transduction to generate GSO47, GSO71, and GSO72, respectively.

RNA isolation. Cultures were grown under aeration at 37°C in LB (Luria-Bertani) rich medium or M63 minimal medium supplemented with 2 mg of glucose and 20 μg of vitamin B_1 per ml. The cell pellet from 25 ml of culture grown to an optical density at 600 nm of 0.2 to 0.3 was resuspended in 1 ml of TRIZOL (Gibco BRL). All subsequent purification steps were carried out according to the TRIZOL reagent manual (based on reference 8). RNA yields were typically 100 to 200 μg.

Primer extension assay. RNA samples were subjected to primer extension assays as described elsewhere (30), using primers specific to *fur* (5′-CAA GTC CCT GGC AAA TAT TG and 5′-TAT TGT TAT CAG TCA TGC GG) and *fldA* (5′-CCA AGC TGT TTT TGA ATC AT).

Northern blotting. The RNA samples (10 μg) were denatured at 65°C in 0.5× Tris-borate-EDTA buffer–70% formamide, separated on 6% urea-polyacrylamide gels, and transferred to nylon membranes by electroblotting. The membranes were probed with the *Eco*RI-*Bam*HI fragment of pGSO97 (^{32}P labeled at the *Eco*RI site) and the *Eco*RI-*Bam*HI fragment of pGSO96 (random primer labeled with ^{32}P).

Protein purification. OxyR protein was purified as described elsewhere (30). Strain XA90 harboring the SoxS overexpression plasmid (pKOXS) was obtained from B. Demple. Crude extracts of cells overexpressing the SoxS protein were prepared as described by Li and Demple (21).

DNase I footprinting. The DNase I footprinting assay of OxyR binding to the *fur* promoter, contained on the *Bam*HI-*Eco*RI fragment of pGSO97, was carried out as described previously (31). The DNase I footprint assay of SoxS binding to the *fldA* promoter, contained on the *Bam*HI-*Hin*dIII fragment of pGSO98, was carried out as described previously (21).

Immunoblot assays. Proteins were separated on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gels and transferred to a nitrocellulose filter by electroblotting. The filter was then probed with a 1:500 dilution of Fur antiserum, provided by M. Vasil. Bound antibody was visualized with rabbit antiserum by using an enhanced chemiluminescence Western blotting kit from Amersham.

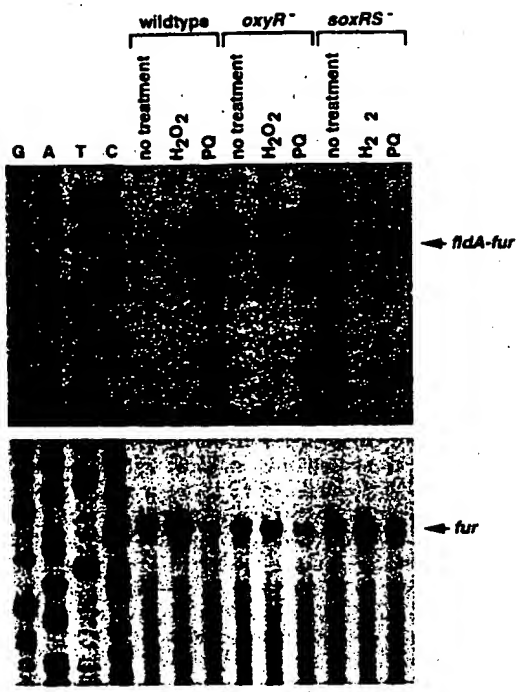

FIG. 2. Primer extension assays of *fur* and *fldA* expression in wild-type, Δ*oxyR*, and Δ*soxRS* strains grown in LB. Exponential-phase cultures were split into three aliquots; one aliquot was left untreated, one was treated with 1 mM hydrogen peroxide, and the third was treated with 0.1 mM paraquat (PQ). The cells were then harvested after 10 min. Total RNA was isolated, and primer extension assays were carried out with primers specific to *fldA* (5′-CCA AGC TGT TTT TGA ATC AT; top) and *fur* (5′-CAA GTC CCT GGC AAA TAT TG; bottom). The neighboring sequencing reactions were carried out with the same primers.

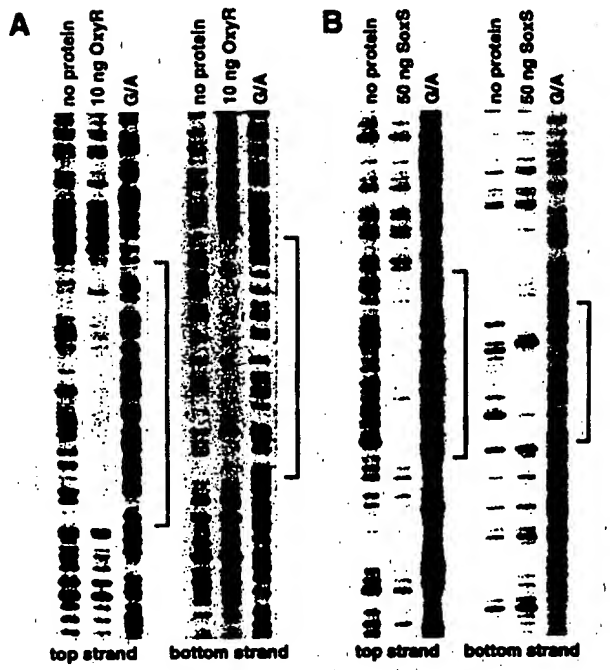

FIG. 3. DNase I footprinting assays of OxyR binding to the *fur* promoter and SoxS binding to the *fldA* promoter. Protected regions on both strands of each promoter are indicated by the brackets. For the OxyR binding to the *fur* promoter, the *Bam*HI-*Eco*RI fragment of pGSO97 was ^{32}P labeled at either the *Bam*HI site (top strand) or the *Eco*RI site (bottom strand), and the DNase I footprinting was carried out as described elsewhere (31). For the SoxS binding to the *fldA* promoter, the *Bam*HI-*Hin*dIII fragment of pGSO98 was ^{32}P labeled at either the *Bam*HI site (top strand) or the *Hin*dIII site (bottom strand), and the DNase I footprinting was carried out as described elsewhere (21). The samples were run in parallel with Maxam-Gilbert G/A sequencing ladders.

The α-Fur antiserum was precleaned as follows. The cell pellet of 20 ml of GSO72 (Δ*fur*::*kan* strain) grown to optical density at 600 nm of 0.2 was resuspended in 100 μl of phosphate-buffered saline, mixed with 6 μl of lysozyme (10 mg/ml), and then frozen and thawed three times. The lysed cells were spread onto a nitrocellulose filter, and the filter was allowed to dry for 10 min. The filter was then incubated with the diluted Fur antiserum for 2 h. After the GSO72 filter was removed, the diluted serum was used to probe the experimental filters. The purified Fur protein was provided by C. Outten and T. O'Halloran.

RESULTS

OxyR activation of *fur*. This work was initiated by a search for additional OxyR binding sites. Rather than carry out traditional genetic or biochemical screens, we used a computational approach based on information theory (26). With this approach, previously identified OxyR binding sites (31) were used as a model to search the *E. coli* genome sequence (5). The computer search indicated an OxyR binding site at coordinate 710103, centered at 234 bp upstream of the start codon of the *fur* gene (Fig. 1). This site has an information content of 14.5 bits, which is higher than those of the *oxyR* (13.9 bits), *dps* (12.3 bits), and *gorA* (11.2 bits) sites but lower than those of the *katG* (19.1 bits), *ahpC* (23.1 bits), and *grxA* (26.0 bits) sites.

To determine whether *fur* is regulated by OxyR, we examined *fur* expression in wild-type and Δ*oxyR*::*kan* mutant strains treated with hydrogen peroxide. Primer extension assays carried out with two different primers showed that *fur* mRNA levels are induced by 1 mM hydrogen peroxide and that this induction is *oxyR* dependent (Fig. 2, bottom, and data not shown). Similar 10-fold induction was observed in rich (LB) and minimal (M63) medium. The start of the *fur* message was mapped to two adjacent G residues located more than 100 bases upstream of the two transcription starts reported by de Lorenzo et al. (10). The RNA sample analyzed in this previ us study was derived from a *fur-lacZ* fusion plasmid carrying only 80 bp of the *fur* promoter. The previously mapped transcription start might correspond to a weak promoter or is an artifact of the reverse transcription reaction.

To test for OxyR binding to the promoter region of *fur*, we carried out DNase I footprinting experiments. As shown in Fig. 3A, the region protected from DNase I digestion directly corresponds to the binding site predicted by information theory (Fig. 1). The OxyR binding site is immediately upstream f the −35 region of the promoter, an arrangement that has been observed at other OxyR-activated promoters (31).

SoxRS activation of *fldA-fur*. The results described above and the intimate relationship between oxidative stress and iron metabolism prompted us to investigate the effect of paraquat treatment on expression of the *fur* gene. We observed a decrease rather than an increase of the main primer extension product detected with the *fur*-specific primer (Fig. 2, bottom). However, we noticed the presence of a longer extension product induced by paraquat in a *soxRS*-dependent fashion. We mapped the start of this extension product to the promoter region of the *fldA* gene, which is directly upstream of and in the same orientation as the *fur* gene (Fig. 2, top). Treatment with 0.1 mM paraquat led to a similar 10-fold induction of the *fldA* transcript in both rich and minimal media (data not shown).

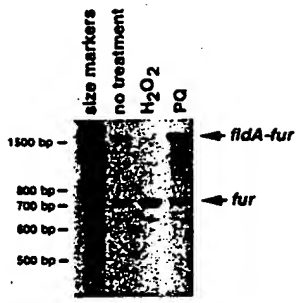

FIG. 4. Northern blot of total cellular RNA isolated from wild-type cells grown in LB which were left untreated or were exposed to 1 mM hydrogen peroxide or 0.1 mM paraquat (PQ). The blot was probed with a ^{32}P-labeled fragment carrying the *fur* promoter (pGSO97). A ^{32}P-labeled 100-bp DNA ladder was electrophoresed alongside the RNA to determine the approximate sizes of the RNA species. Correspondingly, the lengths of the upper and lower transcripts are estimated to be 1.5 and 0.7 kb, respectively.

To determine whether the long extension product is from an mRNA that encompasses both *fldA* and *fur*, we carried out Northern blot analysis on the RNA samples used for the primer extension assays shown in Fig. 2. A transcript that is detected by both *fldA* (data not shown) and *fur* probes (Fig. 4) is induced by paraquat. Interestingly, hydrogen peroxide treatment leads to a significant decrease in the levels of the *fldA-fur* transcript while paraquat treatment leads to a slight decrease in the levels of the *fur* transcript. We do not know the reasons for this regulation.

To test for SoxS binding to the promoter region of *fldA*, we again carried out DNase I footprinting assays (Fig. 3B). The region protected by SoxS spans 25 bases on the coding strand (Fig. 1 and 3B) and contains a GCAC sequence which matches the previously determined SoxS binding motif (22).

High levels of Fur protein. To assess whether the OxyR and SoxRS induction of *fur* transcription leads to changes in Fur protein levels, we carried out immunoblot analysis. As shown in Fig. 5A, both hydrogen peroxide treatment and paraquat treatment led to a twofold increase in the intracellular Fur protein levels within 30 min. By comparing the cellular Fur

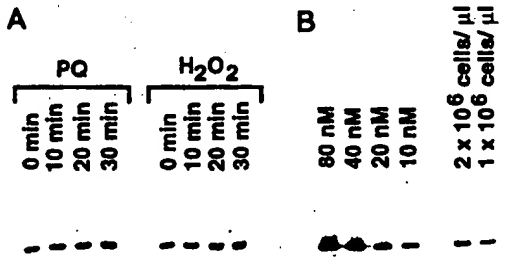

FIG. 5. Immunoblot analysis of Fur protein levels. (A) Wild-type cells (MC4100) grown to exponential phase in LB were treated with 0.2 mM hydrogen peroxide or 0.1 mM paraquat (PQ), and samples were taken at the indicated times. The cell pellets were then resuspended in protein loading buffer to give a final concentration of 2×10^6 cells/μl. An aliquot (5 μl) of each sample was loaded on an SDS-polyacrylamide gel and probed with Fur antiserum. (B). Purified Fur protein was suspended in protein loading buffer to give final concentrations of 80, 40, 20, and 10 nM. Aliquots (5 μl) of each purified Fur sample together with aliquots (5 and 2.5 μl) of the sample from the untreated cells were loaded in the indicated lanes of an SDS-polyacrylamide gel and probed with Fur antiserum.

levels with defined amounts f purified Fur protein, we estimate that the intracellular Fur concentration in nontreated cells during exponential growth to be approximately 5,000 molecules/cell (Fig. 5B). Thus, oxidative stress leads to a concentration of approximately 10,000 Fur m lecules/cell. These levels are similar to the 2,500 Fur molecules/cell determined for *Vibrio cholerae* (33).

DISCUSSION

We have shown that the primary regulators of the *E. coli* response to oxidative stress, OxyR and SoxRS, modulate the levels of the ferric uptake regulator Fur. OxyR, which senses elevated levels of hydrogen peroxide, binds to the *fur* promoter and induces the expression of a transcript encoding Fur. SoxR and SoxS, which modulate the response to superoxide-generating compounds, activate the expression of a transcript encoding both flavodoxin and Fur. This is achieved by SoxS binding to the promoter region of *fldA*. The induction of *fur* transcription by OxyR and SoxRS leads to an increase in Fur protein concentration. We propose that this regulatory network may also exist in other bacterial organisms, especially since Fur is ubiquitous among prokaryotes. We have observed that the *fldA-fur* gene arrangement is conserved in *Klebsiella pneumoniae* (1), *Haemophilus influenzae*, *Yersinia pestis*, and *Actinobacillus actinomycetemcomitans*.

It is well documented that a major portion of the hydrogen peroxide toxicity in *E. coli* can be attributed to DNA damage which is caused by hydroxyl radicals generated by hydrogen peroxide and intracellular iron via the Fenton reaction (18). In addition, recent work has demonstrated that superoxide toxicity is mainly due to its role in accelerating the Fenton reaction (20, 24). Superoxide attacks iron-sulfur proteins, leading to the release of iron that can participate in the Fenton reaction. Since the toxicities of both hydrogen peroxide and superoxide are exacerbated by iron, Fur induction by both OxyR and SoxRS reflects the chemistry of oxidative stress. The finding that Fur is induced by oxidative stress is also consistent with the observation that Fur provides protection against oxidative damage and mutagenesis (32). However, the exact consequences of the twofold increase in Fur levels are unclear. Possibly, new Fur synthesis is required to replace Fur that is damaged by oxidants reacting with metals bound by the repressor. The increase in Fur levels may also be required for increased repression of iron uptake.

In general, the very high levels of the Fur protein raise questions as to the role of this transcription factor. We have found that there are approximately 5,000 Fur molecules/cell in exponentially growing *E. coli* cultures and approximately 10,000 Fur molecules/cell after oxidative stress. The levels that we have determined for *E. coli* are comparable with the 2,500 Fur molecules/cell reported for *V. cholerae* (33) but much higher than the levels of other transcription factors. For example, there are estimated to be 10 to 20 copies of the LacI repressor and 50 to 300 copies of the Trp repressor per cell (12, 19). One possible reason for the high concentration of Fur protein is that there are many predicted Fur sites in the *E. coli* genome (20a). The regulation of iron uptake genes, however, may not be the sole function of Fur. Possibly, Fur sequesters iron in order to reduce the levels of DNA-bound iron. This function would be similar to a function ascribed to the ferritin-like, nonspecific DNA binding protein Dps (13), whose expression is also regulated by OxyR. Another role of Fur may be to catalyze the breakdown of hydrogen peroxide. The dismutation of hydrogen peroxide is a thermodynamically favorable reaction ($\Delta E = +1.06$ V) and could be catalyzed by the Fur-bound

iron cycling between the Fe^{2+} and Fe^{3+} oxidation states. Roles in addition t transcriptional regulation should be considered in further studies f Fur function.

We have also shown that the expression of flavodoxin A is coinduced with Fur when cells are treated with paraquat. Flavodoxin together with ferredoxin/flavodoxin-NADP$^+$ reductase (encoded by *fpr*) functions as a versatile reduction system f r many metalloproteins. It is required for the conversion of dethiobiotin to biotin and the activation of anaerobic ribonucleotide reductase, anaerobic pyruvate formate-lyase, and vitamin B_{12}-dependent methionine synthase (reviewed in reference 17). Interestingly, the expression of the ferredoxin/flavodoxin-NADP$^+$ reductase is also regulated by SoxRS (23). In addition, *fpr* deletion strains are hypersensitive to paraquat killing and ferredoxin/flavodoxin-NADP$^+$ reductase-overexpressing strains are resistant (4). However, the roles of flavodoxin and the ferredoxin/flavodoxin-NADP$^+$ reductase in protecting against oxidative stress are not clear. We speculate that one role of the flavodoxin reduction system might be to keep Fe-S clusters reduced and thus resistant to superoxide attack. Another possible role of flavodoxin and the ferredoxin/flavodoxin-NADP$^+$ reductase may be to modulate the redox state of the SoxR protein. In fact, compared to a wild-type control strain, we have observed stronger initial *soxS* induction followed by a faster decay of *soxS* levels after paraquat treatment of an flavodoxin overexpression strain (data not shown).

Our findings, together with the report that Fur binds to its own promoter (10), suggest that the regulation of *fur* expression is complex. This conclusion is reinforced by additional computation searches which indicate that the cyclic AMP receptor protein and MarA regulator may also bind in the *fldA-fur* region (28). Our results also illustrate the coordination between different regulatory pathways in the response to oxidative stress. Given the wide range of oxidative damage to the cell, it is conceivable that DNA repair, protein degradation, metabolic energy generation, cell division, and other cellular activities are all coordinately regulated. Together, these activities form an oxidative stress response network. Mapping out the connectivity of this network and quantitatively modelling the regulation is one important direction for future studies of cellular defenses against oxidative stress.

ACKNOWLEDGMENTS

We thank B. Demple and L. Rosner for strains, T. O'Halloran and C. Outten for purified Fur, and M. Vasil for Fur antiserum. We also appreciate the editorial comments of T. O'Halloran and D. Touati.

M.Z. acknowledges a postdoctoral fellowship from American Cancer Society.

REFERENCES

1. **Achenbach, L. A., and E. G. Genova.** 1997. Transcriptional regulation of a second flavodoxin gene from *Klebsiella pneumoniae*. Gene 194:235–240.
2. **Bagg, A., and J. B. Neilands.** 1985. Mapping of a mutation affecting regulation of iron uptake systems in *Escherichia coli* K-12. J. Bacteriol. 161:450–453.
3. **Bagg, A., and J. B. Neilands.** 1987. Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry 26:5471–5477.
4. **Bianchi, V., E. Haggård-Ljungquist, E. Pontis, and P. Reichard.** 1995. Interruption of the ferredoxin (flavodoxin) NADP+ oxidoreductase gene of *Escherichia coli* does not affect anaerobic growth but increases sensitivity to paraquat. J. Bacteriol. 177:4528–4531.
5. **Blattner, F. R., G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, et al.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277:1453–1474.
6. **Braun, V.** 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. Biol. Chem. 378:779–786.
7. **Braun, V., K. Hantke, and W. Köster.** 1998. Bacterial iron transport: mechanisms, genetics, and regulation, p. 67–145. *In* A. Sigel and H. Sigel (ed.), Metal ions in biological systems. Marcel Dekker, New York, N.Y.
8. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.
9. **De Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. 169:2624–2630.
10. **de Lorenzo, V., M. Herrero, F. Giovannini, and J. B. Neilands.** 1988. Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *Escherichia coli*. Eur. J. Biochem. 173:537–546.
11. **Gaudu, P., N. Moon, and B. Weiss.** 1997. Regulation of the *soxRS* oxidative stress regulon. Reversible oxidation of the Fe-S centers of SoxR *in vivo*. J. Biol. Chem. 272:5082–5086.
12. **Gilbert, W., and B. Müller-Hill.** 1966. Isolation of the *lac* repressor. Proc. Natl. Acad. Sci. USA 56:1891–1898.
13. **Grant, R. A., D. J. Filman, S. E. Finkel, R. Kolter, and J. M. Hogle.** 1998. The crystal structure of Dps, a ferritin homolog that binds and protects DNA. Nat. Struct. Biol. 5:294–303.
14. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 87:6181–6185.
15. **Hantke, K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol. Gen. Genet. 182:288–292.
16. **Hidalgo, E., H. Ding, and B. Demple.** 1997. Redox signal transduction: mutations shifting [2Fe-2S] centers of the SoxR sensor-regulator to the oxidized form. Cell 88:121–129.
17. **Hoover, D. M., and M. L. Ludwig.** 1997. A flavodoxin that is required for enzyme activation: the structure of oxidized flavodoxin from *Escherichia coli* at 1.8 Å resolution. Protein Sci. 6:2525–2537.
18. **Imlay, J. A., S. M. Chin, and S. Linn.** 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. Science 240:640–642.
19. **Kelley, R. L., and C. Yanofsky.** 1982. *trp* aporepressor production is controlled by autogenous regulation and inefficient translation. Proc. Natl. Acad. Sci. USA 79:3120–3124.
20. **Keyer, K., and J. A. Imlay.** 1996. Superoxide accelerates DNA damage by elevating free-iron levels. Proc. Natl. Acad. Sci. USA 93:13635–13640.
20a. **Lewis, K., M. Zheng, G. Storz, and T. D. Schneider.** Unpublished observation.
21. **Li, Z., and B. Demple.** 1994. SoxS, an activator of superoxide stress genes in *Escherichia coli*. Purification and interaction with DNA. J. Biol. Chem. 269:18371–18377.
22. **Li, Z., and B. Demple.** 1996. Sequence specificity for DNA binding by *Escherichia coli* SoxS and Rob proteins. Mol. Microbiol. 20:937–945.
23. **Liochev, S. I., A. Hausladen, W. F. Beyer, and I. Fridovich.** 1994. NADPH: ferredoxin oxidoreductase acts as a paraquat diaphorase and is a member of the *soxRS* regulon. Proc. Natl. Acad. Sci. USA 91:1328–1331.
24. **McCormick, M. L., G. R. Buettner, and B. E. Britigan.** 1998. Endogenous superoxide dismutase levels regulate iron-dependent hydroxyl radical formation in *Escherichia coli* exposed to hydrogen peroxide. J. Bacteriol. 180:622–625.
25. **Schneider, T. D.** 1996. Reading of DNA sequence logos: prediction of major groove binding by information theory. Methods Enzymol. 274:445–455.
26. **Schneider, T. D.** 1997. Information content of individual genetic sequences. J. Theor. Biol. 189:427–441.
27. **Schneider, T. D.** 1997. Sequence walkers: a graphical method to display how binding proteins interact with DNA or RNA sequences. Nucleic Acids Res. 25:4408–4415.
28. **Schneider, T. D.** 30 March 1999, posting date. Fur region lister maps. [Online.] http://www.lecb.ncifcrf.gov/~toms/paper/oxyrfur/. [16 June 1999, last date accessed.]
29. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. 2:188–194.
30. **Storz, G., and S. Altuvia.** 1994. OxyR regulon. Methods Enzymol. 234:217–223.
31. **Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell 78:897–909.
32. **Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despied.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. 177:2305–2314.
33. **Watnick, P. I., T. Eto, H. Takahashi, and S. B. Calderwood.** 1997. Purification of *Vibrio cholerae* Fur and estimation of its intracellular abundance by antibody sandwich enzyme-linked immunosorbent assay. J. Bacteriol. 179:243–247.
34. **Zheng, M., F. Åslund, and G. Storz.** 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science 279:1718–1721.